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INNATE IMMUNE FACTORS IN RECURRENT AND PERSISTENT INFECTIONS OF THE LOWER FEMALE GENITAL TRACT

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Innate Immune Factors in Recurrent and Persistent Infections of the Lower Female Genital Tract

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my children with love

ABSTRACT

Background: Infections of the female genital tract are common and have severe impact on the quality of life and sexual health of affected women. The innate immune system is crucial and is involved in the defense of infections. Understanding innate immunity of the vaginal and cervical mucosa is important to enable development of future preventive and therapeutic strategies for genital infections.

Objective: The aim of this thesis was to investigate innate immune factors in women with recurrent candida vulvovaginitis (RVVC) and high grade squamous intraepithelial lesions (HSIL) of the cervix, induced by human papilloma virus (HPV). This in order to contribute to the understanding of the innate immune response in these two common infections of the lower female genital tract.

Material and Methods: Clinical examinations, measurements of intravaginal nitric oxide (NO) levels and vaginal biopsies were performed in 28 patients with RVVC and 31 healthy controls. Cervical biopsies and vaginal lavage were collected from 19 patients with HPV induced HSIL and 14 controls. Immunohistochemistry (IHC), enzyme-linked immunosorbent assay (ELISA), western blot and reverse transcriptase real time polymerase chain reaction (PCR)) were used to identify and quantify inducible nitric oxide synthase (iNOS), antimicrobial proteins, cytokines and encoding genes. Adhesion and binding assays were performed to evaluate the adhesive capacity of candida and to demonstrate the binding between candida and the antimicrobial protein psoriasin. Transmission electron microscopy was conducted to measure the cell wall thickness in *C. albicans* affected by psoriasin. To evaluate a new treatment strategy for RVVC, the effect of chlorhexidine digluconate and fluconazole on *C. albicans* eradication and biofilm was investigated in RVVC and commensal strains, using the crystal violet method and viable count.

Results: NO levels were significantly higher in patients during acute infection compared to controls. Levels decreased after fluconazole treatment but remained higher than in controls. Furthermore, increased expression of iNOS was observed in the epithelial basal layer in patients both before and after treatment. There were positive correlations between NO levels, clinical symptoms and examination scores. Findings indicated that *C. albicans* induces production of psoriasin during mucosal candida infection. Psoriasin was shown to interact with β -glucan in the candida cell wall and inhibit candida adhesion to surfaces. In HPV induced HSIL, psoriasin expression and protein levels increased significantly after surgical treatment and reached similar levels as in controls. The mRNA expression of the pro-inflammatory cytokine IL-8 was higher before treatment and restored to the same levels as in controls six months after lesions were excised. Chlorhexidine digluconate prevented new biofilm formation and reduced already established *C. albicans* biofilm. Moreover, the number of candida cells in both planktonic state and within the biofilm were significantly decreased. Although fluconazole reduced the growth of *C. albicans*, no effect was observed on biofilm or candida cells in the biofilm.

Conclusions: Several factors of the innate immune system are involved in the local immune response to RVVC and cervical HPV induced HSIL. The mucosal inflammation during an acute episode of RVVC is well demonstrated by the pronounced increase of vaginal NO, also corresponding to intensity of symptoms. The AMP psoriasin was upregulated in RVVC and was found to have an anti-adhesive effect on *C. albicans*, a result contributing to the understanding of host-pathogen interaction during candida infections. Although fluconazole is the first line treatment of RVVC, the effect is not always satisfactory. According to our results, one explanation could be an inability of fluconazole to dissolve biofilm and eliminate candida cells within the biofilm. Instead local application of chlorhexidine digluconate might be an alternative prophylactic and treatment strategy that inhibits biofilm formation and eradicates *C. albicans* both in planktonic phase and within biofilm. Alterations in expression of antimicrobial peptides and pro-inflammatory markers in HPV induced cervical HSIL demonstrate activation of innate immunity also in premalignant lesions, however the importance of these results needs further exploration.

Keywords: *C. albicans*, recurrent vulvovaginal candidiasis (RVVC), nitric oxide (NO), inducible nitric oxide synthase, (iNOS), antimicrobial peptides (AMP), psoriasin, β -glucan, cervical dysplasia, high-grade squamous intraepithelial lesions (HSIL), human papilloma virus (HPV), biofilm, chlorhexidine digluconate (CHG)

LIST OF SCIENTIFIC PAPERS

- I. **Cathrin Alvendal**, Sophia Ehrström, Annelie Brauner, Jon O. Lundberg, Nina Bohm-Starke
Elevated nitric oxide in recurrent vulvovaginal candidiasis – association to clinical findings
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- II. Annelie Brauner, **Cathrin Alvendal**, Milan Chromek, Konrad Stopsack, Sophia Ehrström, Jens M Schröder, Nina Bohm-Starke
Psoriasin, a novel anti-*Candida albicans* adhesion
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- III. **Cathrin Alvendal**, Witchuda Kamolvit, Siegfried Wagner, Annelie Brauner, Nina Bohm-Starke
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- IV. **Cathrin Alvendal**, Soumitra Mohanty, Nina Bohm Starke, Annelie Brauner
New treatment strategy to prevent recurrences of vulvovaginal candidiasis
In manuscript

CONTENTS

1	INTRODUCTION	1
1.1	Infections in the lower female genital tract	1
1.1.1	Vulvovaginal candidiasis	1
1.1.2	Biofilm formation and persister cells	6
1.1.3	HPV	7
1.2	The immune defense of the female genital tract	10
1.2.1	Laktobacillus	10
1.2.2	Innate immune defense	11
1.2.3	Adaptive immune defense	16
1.2.4	Inflammation and carcinogenesis	16
2	AIMS	19
3	PARTICIPANTS.....	20
3.1	Subjects and Ethics.....	20
3.1.1	Patients with RVVC.....	20
3.1.2	Healthy control women with no history of RVVC	20
3.1.3	Patients with HSIL	21
3.1.4	Healthy controls with no HSIL.....	22
4	METHODS.....	23
4.1	Questionnaires	23
4.2	NO-measurement.....	23
4.3	Examination and sampling.....	24
4.4	Identification and isolation of <i>C. albicans</i>	24
4.5	Immunohistochemistry.....	25
4.6	Total RNA extraction and real-time PCR analysis	25
4.7	Western blot.....	26
4.8	Enzyme-linked immunosorbent assays (ELISA)	26
4.9	Chemicals and reagents.....	26
4.10	Candida adhesion assay.....	27
4.11	Psoriasin – candida binding assays	27
4.12	Cell culture and cell infection	27
4.13	Transmission electron microscopy	28
4.14	Microtiter method to measure <i>C. albicans</i> biofilm	28
4.15	Crystal violet method to analyze the effect of chlorhexidine digluconate and fluconazole on biofilm and <i>C. albicans</i>	28
4.16	Enumeration of <i>C. albicans</i> in mature biofilm	29
4.17	Statistics	29
5	RESULTS.....	30
5.1	Study I: Nitric oxide in recurrent vulvovaginal candidiasis.....	30
5.1.1	Clinical background	30
5.1.2	Symptoms and clinical findings	30
5.1.3	NO levels in patients and controls	31

5.1.4	iNOS expression.....	33
5.2	Study II: Psoriasin, a novel anti- <i>Candida albicans</i> adhesin.....	34
5.2.1	Clinical background	34
5.2.2	Expression of antimicrobials proteins and peptides in the vaginal epithelium.	34
5.2.3	Psoriasin interacts with β -glucan in the <i>Candida</i> cell wall	36
5.2.4	Psoriasin inhibits <i>C. albicans</i> adhesion to surfaces.....	37
5.2.5	Epithelial immune response to <i>C.albicans</i> is enhanced by psoriasin.....	39
5.3	Study III: Psoriasin expression in cervical HSIL	40
5.3.1	Clinical background	40
5.3.2	The expression of psoriasin	40
5.3.3	The expression of IL-8.....	42
5.4	Study IV: New treatment strategy of RVVC.....	43
5.4.1	Participants and samples	43
5.4.2	Fluconazole has limited effect on RVVC strains in mature biofilm	43
5.4.3	Chlorhexidine digluconate dissolves established biofilm of RVVC strains and inhibits candida growth	44
5.4.4	Biofilm formation can be inhibited by chlorhexidine digluconate in RVVC strains	44
5.4.5	Commensal <i>C. albicans</i> growth and biofilm is affected by chlorhexidine digluconate	45
6	DISCUSSION	46
6.1	Discussion of the results	46
6.1.1	NO levels, symptoms and clinical findings.....	46
6.1.2	Psoriasin in <i>C. albicans</i> infections.....	47
6.1.3	Antimicrobial peptides in HSIL.....	49
6.1.4	Biofilm and antifungal strategies.....	50
6.2	Methodological Considerations	51
6.2.1	Participants	51
6.2.2	NO measurement.....	52
6.2.3	Examination and sampling.....	52
6.2.4	Treatment.....	53
6.2.5	Chemicals and reagents.....	53
6.3	Future perspectives and Clinical implications.....	54
7	CONCLUSIONS	56
8	POPULÄRVETENSKAPLIG SAMMANFATTNING	57
9	ACKNOWLEDGEMENTS.....	61
10	REFERENCES	65

LIST OF ABBREVIATIONS

AIN	Anal intraepithelial neoplasia
AMP	Antimicrobial peptides
BH4	Tetrahydrobiopterin
BV	Bacterial vaginosis
CaM	Calmodulin
CFU	Colony forming unit
CHG	Chlorhexidine digluconate
CLR	C-type lectin receptor
COC	Combined oral contraceptive
COX	Cyclooxygenase
CSF	Colony stimulating factor
ELISA	Enzyme-linked immunosorbent assay
FAD	Flavin adenine dinucleotide
FLZ	Fluconazole
FMN	Flavin mononucleotide
FGT	Female genital tract
FRT	Female reproductive tract
H ₂ O ₂	Hydrogen peroxide
HBD	Human beta defensin
HIV	Human immunodeficiency virus
HNP	Human neutrophil peptide
HPV	Human papilloma virus
HSIL	High grade squamous intraepithelial lesions
HSV	Herpes simplex virus
IHC	Immunohistochemistry
IL	Interleukin
iNOS	Inducible nitric oxide synthase

LSIL	Low grade squamous intraepithelial lesions
MIC	Minimal inhibitory concentration
NO	Nitric oxide
OC	Oral contraceptives
OSCC	Oral squamous cell carcinoma
OPSCC	Oropharyngeal squamous cell carcinoma
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyd
PRR	Pattern recognition receptors
PVD	Provoked vestibulodynia
ROS	Reactive oxygen species
RVVC	Recurrent vulvovaginal candidiasis
SCC	Squamous cell carcinomas
STI	Sexual transmitted infection
TGF	Transforming growth factor
TLR	Toll like receptor
VVC	Vulvovaginal candidiasis
TLR	Toll like receptor
TZ	Transformation zone
VFS	Vaginal fluid simulant
YPD	Yeast peptone dextrose

1 INTRODUCTION

1.1 INFECTIONS IN THE LOWER FEMALE GENITAL TRACT

Infections of the female genital tract are common and cause a large proportion of visits to gynecological clinics worldwide. The infections have a severe impact on the sexual health and quality of life in the affected women and leads to significant health care costs [1].

Several different types of bacteria, virus, fungi and parasites infect the female genital tract (FGT). Infections can be recurrent or persistent and cause long-term suffering. Common pathogens include *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Treponema pallidum*, *Mycoplasma genitalium*, *Gardnerella vaginalis*, *Mycoplasma hominis*, human papilloma virus (HPV), herpes simplex virus (HSV), human immunodeficiency virus (HIV), candida species and *Trichomonas vaginalis*.

The main site of infection varies due to differing susceptibility of the infecting microorganisms in various regions of the FGT. *Candida albicans* and *Trichomonas vaginalis* colonize the vagina whereas the endocervix and transformation zone of the cervix is vulnerable to infection by *Chlamydia trachomatis*, *Neisseria gonorrhea*, *Mycoplasma genitalum* and oncogenic strains of HPV [2-6]. Vaginitis is caused by infection and inflammation of the vaginal mucosa. In cervicitis the infection affects the cervical mucosa, mainly the columnar epithelial cells, but it may also cause visible changes to the ectocervix whose squamous epithelium is continuous with the vaginal epithelium. Vulvitis is an infection or inflammation of the vulvar skin and mucosa [7].

Symptoms of infections differ but there are several similarities. The most common symptoms are excessive vaginal discharge, erythema, pruritus, pain, dyspareunia, discomfort during urination and mild bleeding. Most infections, but not all, are sexually transmitted and there is a clear association between bacterial vaginosis (BV) and an increased risk of incurring infections such as HIV and HSV-2 [8].

This thesis focus on factors of the innate immune defense during recurrent vulvovaginal candidiasis and HPV induced cervical intraepithelial lesions (CIN). These infections have major differences in their clinical features and consequences. Candida causes numerous local symptoms with severe inflammation of the infected tissue [9], while HPV mostly is a quiet, non-symptomatic infection but with a potential to induce malignant transformation of the affected tissue [10].

1.1.1 Vulvovaginal candidiasis

Candida is a commensal yeast organism present in the vaginal flora of about 20% of asymptomatic healthy women during their reproductive years [9, 11]. The lifetime incidence of sporadic vulvovaginal candidiasis (VVC) is estimated to 75% and 40-50% of women will experience recurrence [11, 12]. The rate of infections decreases after menopause. Most

women who suffer from sporadic infections are healthy without well-known risk factors such as pregnancy, use of antibiotics, diabetes mellitus and immune deficiency.

Clinical manifestations are pruritus, vaginal discharge, soreness, dysuria and dyspareunia. On clinical examination there are prominent signs of inflammation with erythema and edema in the vulvovaginal mucosa and an adherent off-white discharge [13]. These symptoms and signs are non-specific and diagnosis of VVC needs further verification. Vaginal pH is not affected by VVC and the finding of $\text{pH} \leq 4.5$ helps to exclude bacterial vaginosis (BV). Direct microscopic examination of vaginal secretions is an important diagnostic tool. A wet mount with saline can identify the presence of yeast cells or hyphae/pseudohyphae and rule out the presence of motile trichomonads and clue cells revealing BV. A 10% potassium hydroxide preparation might help to identify fungal elements. However, microscopy has a sensitivity of 50% at the best [14] and therefore additional vaginal yeast culture is recommended in women with symptoms and normal pH and negative microscopy [14].

1.1.1.1 Recurrent vulvovaginal candidiasis

Recurrent vulvovaginal candidiasis (RVVC) is defined as VVC in excess of 3-4 times per year [15, 16] and occurs in 6-9% of premenopausal women [16, 17]. Little is known about risk factors and mechanisms leading to relapses in otherwise healthy women. However, the causation is likely multifactorial and factors such as contraceptives, sexual behavior, candida virulence, genetic factors and chronic stress have been reported [16, 18-21]. A combination of ineffective local immune response and specific virulence factors of pathogenic candida might be contributing factors. It has been proposed that certain candida strains may persist, providing a reservoir for relapsing infections [22-24]. Patients with RVVC exhibit normal systemic cell-mediated immune responses to candida [25-27]. However, impaired or changed vaginal innate immune response predisposing to RVVC has been proposed [11, 26]. In most patients with RVVC no triggering factor is identified.

Symptoms of RVVC are mainly the same as in VVC with soreness, pruritus and dyspareunia. However, the clinical findings may differ and signs of vulvitis often dominate with dry skin, excoriations and fissures. These more or less chronic symptoms severely affect the patients' sexual health and quality of life [1, 9]. The repeated inflammatory tissue response might be a trigger for chronic vulvar pain conditions developing over time [28, 29]. A vaginal culture is important to obtain a correct diagnosis in these patients. If the initial tests are negative, it can be helpful to provide patients with culture swabs with transport medium for self-sampling at home during a symptomatic relapse.

1.1.1.2 Candida species

C. albicans is the most common pathogen in women with VVC (70 to 95%), followed by *C. glabrata* (7 to 16%) [11, 12]. Other species causing VVC are *C. parapsilosis*, *C. tropicalis*, *C. krusei* and *Saccharomyces cerevisiae*. VVC caused by non *albicans* species are clinically impossible to differentiate from VVC caused by *C. albicans* but are often more difficult to

treat. *C. albicans* has the unique ability to evolve through germination into a more invasive mycelial form. This form is capable of invading mucous membranes, possibly explaining its predominant role in the pathogenesis of VVC [30].

In RVVC, *C. albicans* is the major causative agent but there are reports that recurrent infections caused by non albicans species are increasing, most commonly *C. glabrata* [16].

1.1.1.3 *Candida albicans*

C. albicans is a commensal fungus colonizing cutaneous and mucosal surfaces of healthy individuals. The colonization increases during antibiotic treatment due to the antibacterial effect and reduction of the vaginal bacterial flora. However, *C. albicans* can cause diseases in mucosal membranes such as oral and esophageal candidiasis, vulvovaginal candidiasis and skin infections. Immunocompromised patients constitute the highest risk group for severe fungal infections [31]. Moreover, indwelling catheters are risk factors due to the ability of the fungus to adhere and form biofilm to foreign surfaces [32]. *C. albicans* can penetrate mucosal barriers and endothelia and cause candidemia which can develop to disseminated candidiasis when the infection spreads to internal organs [33]. In patients with invasive candidiasis or candidemia the mortality is as high as 40-50%, despite adequate antifungal therapy [31, 33].

C. albicans has the ability to grow as either unicellular yeast cells or as filamentous pseudohyphal or true hyphal forms, *Figure 1*. Pseudohyphae develops when daughter-buds elongates from the yeast cell. The pseudohyphae are distinguishable from hyphae by their constrictions at the site of septation and that they are wider than hyphae. True hyphae form long tube-like filaments with parallel sides without constrictions [34]. Hyphal form has a key role in the infection process by invading epithelial and endothelial cells and is considered to be an important virulence factor of *C. albicans* [33], *Figure 2*. Yeast blastospores is the phenotypic form responsible for transmission from the lower gastrointestinal tract and asymptomatic colonization of the vagina [16].

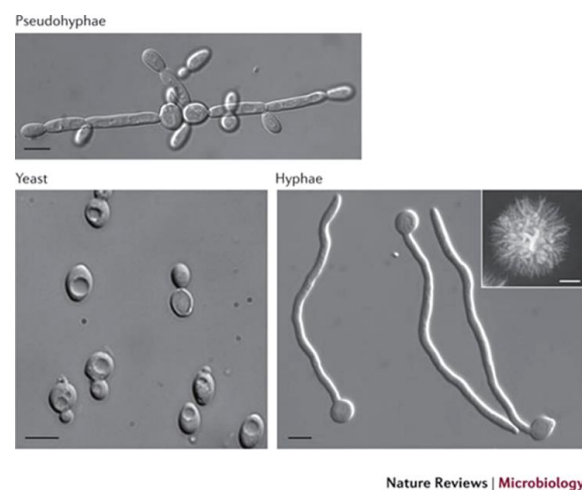


Figure 1. Morphology of C. albicans. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Microbiology [33], copyright (2011).

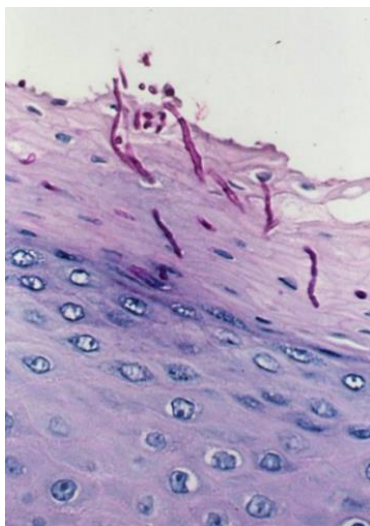


Figure 2. Candida hyphae penetrating the epithelium.

Foto: Eva Rylander

The candida cell wall is constituted by polysaccharides, glycoproteins, enzymes and lipids. The polysaccharides mannan, glucan and chitin are major components of the outer cell wall [35], *Figure 3*. These carbohydrate-containing molecules are recognized by pattern-recognition receptors (PRR) and the innate immune system activates inflammatory responses such as initiation of microbicidal processes by leukocytes, production of reactive oxygen intermediates, inflammatory mediators and cytokines, including tumor necrosis factor α . (TNF- α). The glucans of the candida cell wall are recognized by cells of the innate immune system and are important fungal pathogen associated molecular patterns (PAMPS) [35].

Binding of β -glucan to the C-type lectin dectin-1 receptor, a PPR that mediates induction of pro-inflammatory cytokines in intestinal epithelial cells [36] and keratinocytes [37]. The Dectin-1 receptor also interacts with other PRRs to promote signals inducing immune responses such as production of cytokines and reactive oxygen species (ROS) [38, 39]. Moreover, the size and physical properties of the molecules regulate the immune response where large β -glucan particles stimulate cytokine and ROS production and phagocytosis, while soluble β -glucans bind to but do not activate the Dectin-1 receptor [38, 40, 41].

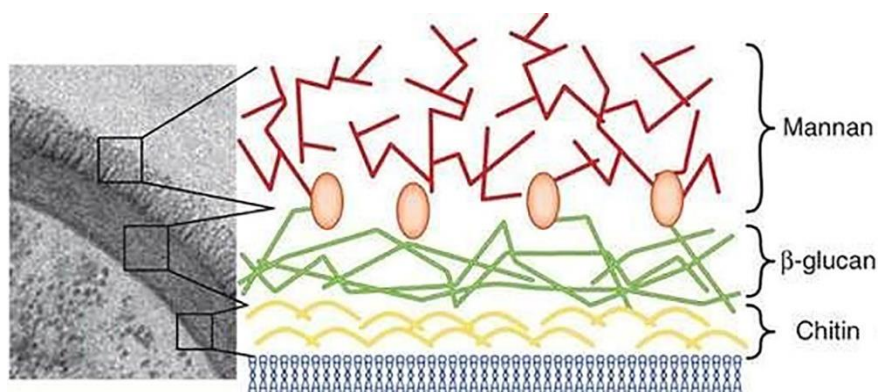


Figure 3. Candida albicans yeast cell wall. Reprinted by permission from Macmillan Publishers Ltd: Nature Immunology [39], copyright (2012).

1.1.1.4 Treatment

Most of the available antifungal agents are not fungicidal. Azoles are fungistatic, thus inhibiting the growth of fungi leading to a clearance of the infection. Most *C. albicans* strains are susceptible to available antimycotic drugs and real resistance of isolates is rare [42-45]. Topical azole agents are available in a variety of formulations and are often sold over the counter for self-treatment. For patients with sporadic VVC, topical treatment will have a curative effect in 80-90% of cases [23]. Oral systemic azole agents achieve comparable therapeutic cure rates in patients with VVC at short term. However, at long-term follow up the oral treatment is more effective in achieving mycological cure [46]. A mild vulvovaginal burning sensation is a common side effect of topical azoles [23, 47]. Using oral administration, local side effects are avoided but instead systemic adverse effects like headache, nausea and abdominal pain might occur in rare cases [23, 43, 47]. The oral azoles are contraindicated during pregnancy. Less frequently used, but still available treatment options are vaginal application of boric-acid, gentian violet and nystatin. They are of no advantages in treatment of trivial *C. albicans* infections but can be useful supplements in complicated cases [16].

For RVVC caused by *C. albicans* long-term treatment with fluconazole is recommended. The most effective regime to date starts with induction of fluconazole 150 mg every 72 hours for 3 doses followed by fluconazole 150 mg once weekly for 6 months [16, 43]. Fluconazole has a half-life time of 25-hours. After administration of a single dose of oral fluconazole 150 mg, concentrations of fluconazole above the minimal inhibitory concentration (MIC) that inhibits the growth of 90 % of candida species isolates (MIC 90) are achieved in vaginal tissue and secretions for 72-96 hours. Due to the pharmacodynamics, weekly administration is adequate [43, 48]. In most cases, there are only a few breakthrough episodes of symptomatic vaginitis during the 6 months' treatment. After cessation of the treatment, 50% of the patients develop a new culture positive outbreak of RVVC within 3-4 months. The cultures usually show the same strain of candida as before treatment and the strain remains sensitive to fluconazole [16]. In those cases, it has been suggested that the azole fails to achieve total eradication of candida organisms. When recurrences occur, a new long-term maintenance treatment with weekly 150 mg fluconazole for further 6-12 months is recommended [16]. However, in some cases it has been reported that long-term usage of antifungals is associated with decreasing sensitivity to fluconazole [49]. Itraconazole can be used as an alternative but has a potential for liver toxicity and is therefore not a first line choice [16].

RVVC with non-albicans candida species are often more complicated to treat due to fluconazole resistance. However, *C. tropicalis* and *C. parapsilosis* are sensitive to fluconazole and can be treated like *C. albicans*. *C. krusei* and *Saccharomyces cerevisiae* are resistant to fluconazole and treatment with clotrimazole, boric acid or nystatin is needed [12]. Also approximately 50% of *C. glabrata* strains are resistant to fluconazole and therefore local treatment with clotrimazole or boric acid is recommended

Patients with RVVC need to avoid dehydration of the mucosal tissue by limiting the use of soap and water. Oil and emollient regime is recommended. Probiotics with exogenous *lactobacillus species* have been suggested to prevent RVVC, however, there are few studies on the efficacy and the results are inconclusive [16, 50]. Although partners often harbor

strains of candida in saliva, rectum or semen it is not proven to be associated with recurrences. Partner treatment is therefore not recommended [51, 52].

Curative treatment for RVVC is difficult to obtain and many patients have symptoms affecting their quality of life for many years. New antifungal strategies are therefore of great importance for both treatment and prevention of these relapsing infections.

1.1.2 Biofilm formation and persister cells

Biofilm is a survival mechanism used by several microorganisms and can be formed on both biotic and abiotic surfaces [53] such as vaginal epithelial cells [54] and intrauterine devices [55, 56]. They are considered to contribute to approximately 80% of microbial infections in the human body [57] and the formation on medical devices such as catheters and heart valves are a major factor causing candidemia with high mortality rate [58, 59]. The biofilm of *C. albicans* is a complex and robust three-dimensional structure containing an exopolymer matrix and a mixture of yeast, pseudohyphae and hyphae [60] *Figure 4*. The biofilm shields candida from the immune system and antifungal treatment [61, 62]. Formation of biofilm is induced when *C. albicans* adhere to the epithelial cells with help of the mannoproteins and adhesins of the fungal cell surface. After adherence, the initiation of micro colonies starts and subsequently the maturation of the biofilm occurs by the production of extracellular polymeric substances and increase of cell numbers. The candida biofilm extracellular matrix is composed of polysaccharides, proteins, phosphorus and uronic acid which scaffolds the biofilm integrity [60, 63]. Although the biofilm is an effective way for microorganisms to circumvent eradication, it does not completely hinder penetration of antifungal treatment. Genes and metabolic pathways might be downregulated in some cells of the biofilm [64, 65]. Many cells in the biofilm are killed by antifungal treatment but a small subpopulation of highly drug tolerant dormant persister cells can survive [62, 66]. On cessation of antimicrobial treatment, the persister cells are dispersed and repopulate the biofilm or colonize new surfaces thereby causing relapse of the infection. However, when these cells are detached from the biofilm they are susceptible to treatment, indicating that persister mechanisms are due to phenotypic changes and not genetic mutations [59, 67]. Persistence is thereby distinct from resistance were the microbes becomes resistant to the given treatment.[68].

Persister cells are acknowledged to play a major role in the recalcitrant nature of chronic infections such as cystic fibrosis pneumonia caused by *Pseudomonas aeruginosa*, tuberculosis caused by *Mycobacterium tuberculosis* and oral candidiasis caused by *Candida albicans* [67].

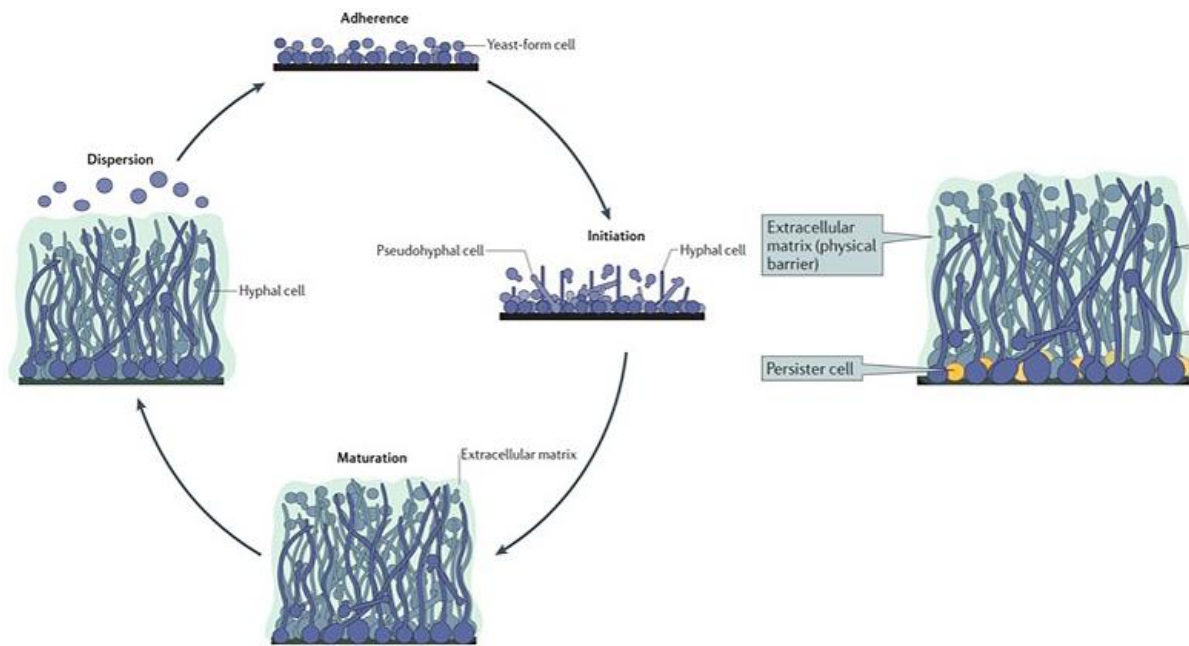


Figure 4. Biofilm formation and persister cells. Reprinted by permission from Springer Nature: *Nature Reviews Microbiology* [65], copyright (2017).

1.1.3 HPV

Human papilloma virus (HPV) are double stranded DNA viruses that infect epithelial cells. A subgroup of which have been proven to be etiologically involved in the development of human cancer. More than 200 types of human papilloma viruses are identified [69]. HPV infection is globally the most widespread sexually transmitted infection (STI) and most sexually active individuals are likely to be exposed during their lifetime [70]. The viruses have predilection for cutaneous and mucosal epithelial tissues. They are divided into groups of low-risk and high-risk HPV depending on their potential to induce carcinogenesis. HPV infect basal cells of stratified squamous epithelium, which can be reached as a result of micro-abrasions and wounds [69]. HPV are intracellular pathogens dependent of the cell machinery of the basal cells in the epithelium to replicate and the HPV DNA becomes incorporated in the host cell DNA [70]. Most infected individuals will effectively eliminate the virus. However, likely due to immune evasion approximately 15% of infections will become persistent. Several possible mechanisms explaining immune evasion have been described [10, 71]. During differentiation, the virus replicates inside the cells and is therefore practically invisible to the host immune system. Virus shedding occurs in the upper layers of the epithelium without causing tissue damage or viremia [10, 70]. Furthermore, HPV downregulates the expression of interferon genes. Interferons are cytokines with potent anti-viral and immunostimulatory effects. [10, 70, 71]. Despite these immune avoiding capacities, a majority of HPV infections resolve with time, mainly thanks to cell mediated immune response [72].

1.1.3.1 HPV infections and related diseases

HPVs show a high grade of tissue tropism and various types of HPV infect particular anatomic areas. The low-risk viruses cause benign hyper-proliferative lesions such as warts among which HPV 6 and 11 cause condyloma acuminatum in the anogenital area [5]. Whereas infections with high risk HPVs can cause invisible or asymptomatic precursor lesions, with the ability to advance to premalignant lesions and invasive cancer and are a major cause of cancers of the cervix, vagina, vulva, anus, penis and oropharynx [73].

Worldwide, cervical cancer is the third most common female cancer with approximately 85% occurring in less developed regions [73]. Nearly 100% of all cervical cancer patients are HPV positive and HPV 16 and 18 are responsible for approximately 70% of cervical carcinomas [70, 72]. Lately, the incidence of HPV positive oropharyngeal squamous cell carcinomas (OPSCC) has increased [74]. It has been speculated that the reason for this development might be alterations in sexual habits with a significant association between HPV-positive OPSCC, early sex debut and number of partners engaged in oral or vaginal sex. Eighty-eight percent of anal cancers are HPV related and populations at increased risk are HIV-positive patients and men having sex with men [75].

1.1.3.2 Cervical HPV lesions

The ectocervix constitutes of stratified squamous epithelium and the endocervix, with the endocervical canal, is lined by a single layer of columnar epithelium. The squamocolumnar junction (SCJ) is the area where these two cell types converge. The transformation zone (TZ) is the area of squamous metaplasia between the current and original squamocolumnar junction. The TZ is characterized by immature squamous epithelium. The proliferating cells of the metaplastic epithelium are susceptible to HPV infection and TZ is where high-risk HPV-associated lesions primarily develop [5, 69].

Of all HPV positive women, approximately 15% develop a persistent infection of the cervical epithelium [76]. Persistent HPV infections may slowly progress to cervical low- and high-grade squamous intraepithelial lesions (LSIL, HSIL) with the potential to develop to invasive squamous cell carcinoma (SCC) [77], *Figure 5*. Cofactors increasing the risk of progression are a compromised immune system and other co-infections such as HSV 2, *Chlamydia trachomatis*, *Neisseria gonorrhoeae* and BV [76, 78, 79]. Prolonged use of oral contraceptives (OC), high parity and smoking are other risk factors for persistent infection [80]. Invasive cancer may develop over several years in a minority of women with HSIL. In Sweden the median age of acquired cervical cancer is 50 years, with an interval ranging from 20 to 85 years [81].

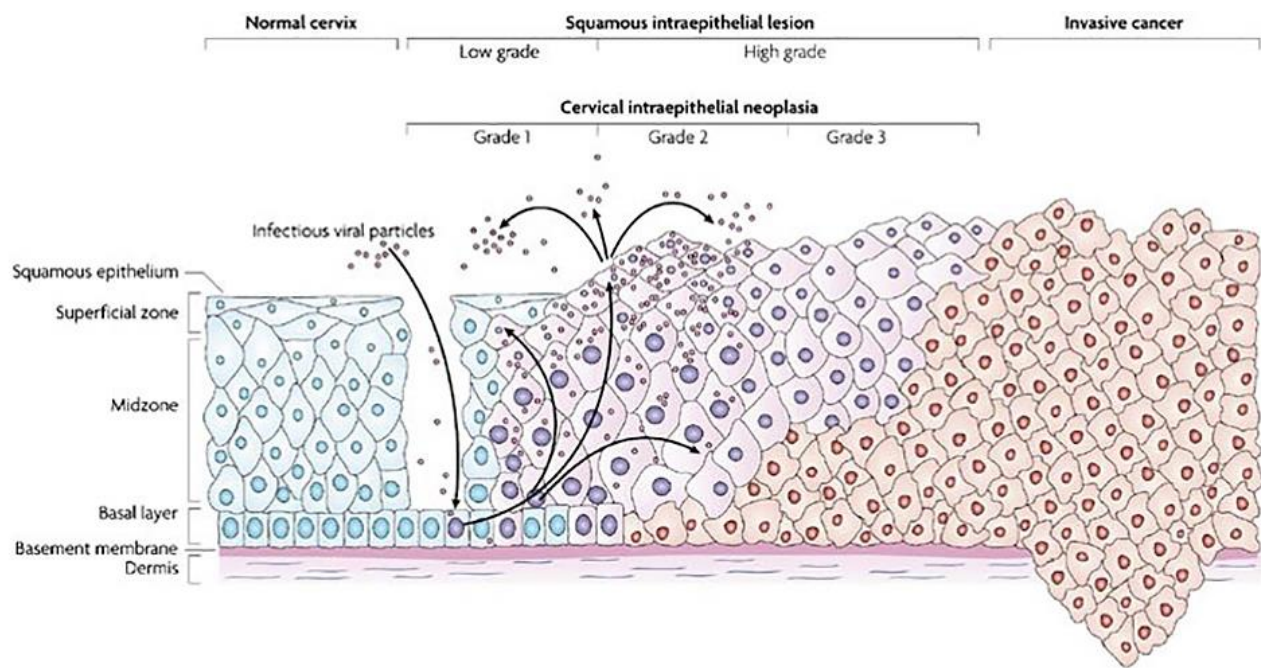


Figure 5. Development of cervical intraepithelial neoplasia. Reprinted by permission from Macmillan Publishers Ltd: *Nature Reviews Cancer* [77], copyright (2007).

1.1.3.3 Screening and diagnosing

Since the early 1960s a national screening program has been in place in Sweden, initially using Pap smears to detect precancerous cervical lesions. The method was described by Dr. Papanicolaou in the early 1940s. Despite a low sensitivity of only approximately 55% to detect a high grade dysplasia (CIN2+) by a single Pap smear (ranged 30- 80%) [82, 83], the screening program has been successful in reducing the incidence and mortality of cervical cancer by 35-70%. In fact, 64% of all cervical cancers and 83% of advanced cases in Sweden were diagnosed in women not attending the screening program [84]. Recently liquid-based cytology (LBC) has largely replaced the Pap smear as sampling method. The cells are collected similarly as for the Pap smear, but instead of primarily distributing the cells on a glass slide, the cells are immersed and rinsed in a collection fluid before fixation and staining. This reduces the number of inadequate samples by eliminating aggravating factors such as blood, mucus and cell deposits and provides an optimal thin layer of cells beneficial for microscopic interpretation. The LBC also enables supplementary testing of high-risk HPV DNA or RNA, increasing the sensitivity for detecting HSIL compared to cytology alone [85]. In the new national Swedish guidelines, HPV analysis is recommended as primary screening method followed by cytology in women older than 30 years [86, 87].

1.1.3.4 Treatment

When primary screening show signs of atypia the patients are referred for colposcopy and biopsy of the TZ or any visible lesion. Treatment is recommended when HSIL is detected or

when the colposcopy is incomplete [86]. The aim of treatment is to excise the high-grade lesion with minimum harm to the cervix. Excision methods used are loop electrosurgical excision procedure (LEEP) or laser conisation and previously cold knife cone biopsy. Excisional treatments are effective and cause low morbidity [88]. For persistent lower grade lesions, destruction by laser ablation or cryotherapy might be an option.

1.1.3.5 HPV Vaccines

A dramatic breakthrough in combating HPV infections was the introduction of HPV virus-like particle (VLP) vaccines. Administered by intramuscular injection the vaccine has a rapid access to local lymph nodes and induce a potent antibody response, with antibody concentrations up to 40 times higher than after a natural infection [89]. The vaccines are well tolerated and effective with almost 100% seroconversion [90]. In addition, available vaccines have shown cross-protection against non-vaccine HPV strands.[91, 92]. In Sweden, HPV vaccination for girls has been included in the national vaccination program since 2010. The vaccine has also been shown to protect adult women from infections and lesions caused by the vaccine type HPVs. Including boys would protect against HPV related cancers and genital warts in men and have beneficial effects for protection of non-vaccinated women and males through increased herd immunity [91].

1.2 THE IMMUNE DEFENSE OF THE FEMALE GENITAL TRACT

The female reproductive tract requires an exceptional regulation of the immune defense. In addition to protection against infectious microbes it must also adjust to a wide range of physiological events including fertilization by allogenic spermatozoa, implantation, pregnancy with an immunologically distinct fetus and parturition. The mucosal system is under hormonal control throughout the menstrual cycle. Protection against potential pathogens in the FRT is provided by a range of activities that can be grouped into two categories, the innate and adaptive immune defense.

1.2.1 Laktobacillus

Lactobacillus spp dominates the vaginal flora in approximately 70% of premenopausal women. The most commonly isolated species are *L. crispatus*, *L. gasseri*, *L. jensenii* and *L. iners* [93]. The lactobacillus dominated microbiota is important for a healthy vaginal ecosystem. The metabolic products lactic acid, hydrogen peroxide (H₂O₂) and bacteriocins are secreted in the cervicovaginal fluid and can directly eradicate or inhibit harmful pathogens. By formation of microcolonies and biofilm that adhere to the epithelial cells, a physical barrier against pathogen adhesion is produced. A low vaginal pH of ≤ 4.5 is one of the main inhibitory mechanism for preventing pathogen colonization of the vaginal tract [94]. This is supported by the fact that risk of STIs acquisition is increased in patients with BV [93].

1.2.2 Innate immune defense

Innate immunity is present even in the in most primitive multicellular organisms. It is an ancient form of immune defense that has evolved by natural selection over millions of years. Genes encoding for these innate immune molecules are inherited from one generation to the next in a stable form. The innate immune system is considered the first line defense and efficiently clear most pathogenic microorganisms. Without a functioning innate immune system we are extremely susceptible to infections.

1.2.2.1 Epithelial cells- the barrier protection

The mucosal lining of the female genital tract, composed by mucus and epithelial cells, provides a physical and immunological barrier to pathogens. The epithelial cells produce mucus, antimicrobial peptides, chemokines and cytokines [95-97]. The cervix, the uterus and the fallopian tubes are lined with columnar epithelium as opposed to vaginal mucosa which consists of a multilayered non keratinized stratified squamous epithelium. The epithelium undergoes differentiation and contains several distinct layers. Exfoliation is an effective way to eliminate pathogens attached to the vaginal mucosa. The epithelial cells contain large cytoplasmic stores of glycogen [98]. Glycogen is a major component of exfoliated cells and serve as a substrate for lactobacilli that produce lactic acid and thereby maintain an acidic pH [99]. Sex hormones influence the thickness of the vaginal epithelium and the glycogen content of the cells. After menopause, with low levels of endogenous estrogen, the vaginal epithelium becomes thinner and glycogen stores are diminished [100, 101]. The physical barrier formed by epithelial cells is dependent on the presence of tight junctions between the cells. In the apical layers of the vaginal and ectocervical epithelia, there is a lack of tight junctions allowing intraepithelial transport of pathogens and immune cells in the spaces between the cells [98, 102, 103]. The epithelial cells express pattern recognition receptors (PRRs), such as Toll like receptors (TLRs) and C-type lectin receptors (CLR) which recognize pathogen associated molecular patterns (PAMPs) on microorganisms. When the PRRs are stimulated by PAMP the epithelial cells respond with secretion of antimicrobial peptides (AMP), cytokines and chemokines.

1.2.2.2 Complement

When a pathogen penetrates the epithelial barrier the complement system is activated. It is a system of soluble proteins constitutively produced by the liver and are present in blood, lymph, extracellular fluids and in vaginal secretions. Complement proteins attach to the surface of bacteria and extracellular virus and make them susceptible for phagocytosis. The complement system consists of more than 30 components [104].

1.2.2.3 Innate immune cells

Circulating monocytes are effector cells in both the innate and the adaptive immune defense. They migrate into tissues where they differentiate into macrophages. Macrophages are long lived phagocytic cells and play a major part in the initiation, maintenance and resolution of

the inflammatory response. Approximately 10% of the leukocytes present in the FRT are macrophages, with high amounts in the endometrial stroma and myometrial connective tissue [102, 105]. The number of macrophages in the endometrial tissue is regulated by estradiol and progesterone levels but in vaginal tissue the numbers of macrophages remain stable throughout the menstrual cycle [102, 105]. Macrophages and dendritic cells are antigen presenting cells. Pathogen exposure and phagocytosis induce antigen presentation, generating responses of the adaptive immune system [104].

Natural killer cells (NK-cells) are the killer lymphocytes of the innate immune defense and provide an early defense against intracellular infections. NK-cells possess cytotoxic activity, produce and secrete cytokines and have the ability to kill tumor cells. Their important role in the FRT innate defense is observed by the increased rate of HSV infections and an increased incidence of cancer in patients with malfunctioning NK cell [102, 105].

Neutrophils circulate in the bloodstream and are attracted to sites of infection by inflammatory mediators. There they interact with the walls of the capillaries, leave the blood stream and move in to the infected area in large numbers. Neutrophils are also present in all tissues of the FRT under healthy conditions and the amount of neutrophils in vaginal tissue is constant during the menstrual cycle [100]. When vaginal candida infection was experimentally induced in healthy women, there was a neutrophil tissue infiltration and inflammation in the symptomatic cases as opposed to asymptomatic cases. This suggests that neutrophils are important for the immuno-pathogenesis and symptomatology of the infectious process [106].

1.2.2.4 Cytokines and Chemokines

Cytokines are small proteins that are produced by immune and epithelial cells and regulate immunity, inflammation and hematopoiesis. They stimulate a potent innate immune response, which create a hostile environment for pathogens and are regulators of the adaptive immune system. They have powerful inflammatory effects localized to the infected tissue and the effects can also be expressed systemically throughout the body. Secretion of cytokines and chemokines lead to interactions between the different cell categories in the FRT. The cytokine levels of cervico-vaginal fluid change during the menstrual cycle and gestation [107, 108].

In response to infection, proinflammatory cytokines, including tumor necrosis factor (TNF), interleukins IL-1, IL-6, IL12 and IL-8 are produced, but once the stimulus is eliminated the inflammatory reaction is naturally controlled and tissue repair is induced [109]. The resolution of inflammation is regulated by several anti-inflammatory mediators like IL4, IL 10, IL-13 and transforming growth factor (TGF)- β [110]. By clearance of dying cells by phagocytes the tissue damage is repaired [111].

Chemokines such as IL-8, produced by cells on the site of infection, are chemoattractants that recruit neutrophils to eradicate microorganisms. They are also involved in cell proliferation, differentiation and angiogenesis [112, 113] .

1.2.2.5 Nitric oxide

The Nobel Prize in Physiology or Medicine 1998 awarded Furchgott, Murad and Ignarro for their discoveries concerning nitric oxide (NO) as a signaling molecule. This was the first discovery that a gas can act as a signal molecule in an organism.

NO is a short lived biologically active free radical gas having an elimination half-life of only a few seconds. The molecule is present in biological tissues and is an active substance involved in numerous physiological processes such as relaxation of smooth muscle cells, neurotransmission, vascular hemostasis and is part of the nonspecific host defense [114]. It has cytotoxic effects against microorganisms and tumor cells [115]. NO is synthesized *in vivo* through the conversion of the amino acid L-arginine to L-citrulline by a process catalyzed by enzymes known as nitric oxide synthases (NOS). The enzymatic reaction requires Nicotinamide adenine dinucleotide phosphate (NADPH) as co-substrate and Heme, Flavin mononucleotide (FMN), Flavin adenine dinucleotide (FAD), Tetrahydrobiopterin (BH₄) and Calmodulin (CaM) as cofactors [116], *Figure 6*.

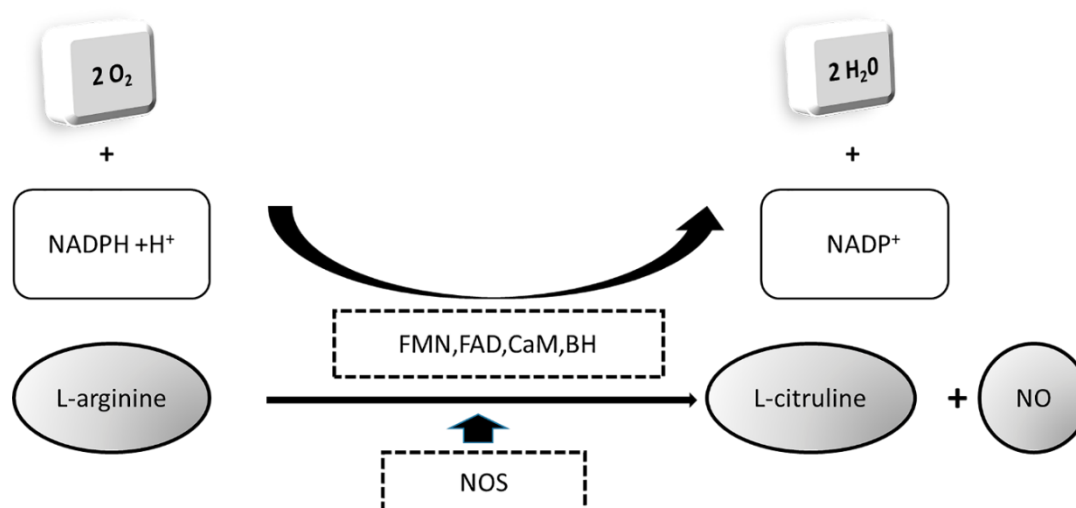


Figure 6. The synthesis of NO.

Three forms of NOS have been described, neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). nNOS and eNOS are named after the tissue in which they first were found. They are constitutively expressed and are calcium dependent. iNOS was originally identified as being inducible by cytokines in macrophages and hepatocytes and is calcium independent [116]. It is widely expressed in various cell types and is induced by bacterial lipopolysaccharides (LPS) and proinflammatory cytokines [117, 118].

Nitric oxide has several significant roles in inflammation. It is a potent vasodilator and plays an essential role in vascular function during the inflammatory process. NO reduces platelet

aggregation and adhesion and acts as a regulator of leukocyte recruitment. It has anti-microbial activity by limiting replication of bacteria, protozoa, fungi and viruses and it has been shown that NO is bactericidal to various strains of bacteria [119, 120]. However, NO can also induce cytotoxic effects in tissues by oxidative injury with cellular and organ dysfunction due to its interaction with oxygen radicals generating peroxynitrite [121]. The role of NO in HIV infection is dichotomous since NO seems to have double functions in the pathogenesis of HIV-1 by both blocking HIV-1 replication and by the NO-induced oxidative stress leading to rapid viral evolution [121].

NO measurement in biological specimen is difficult due to the lability of NO in the presence of oxygen. There are assays that indirectly reveal the presence of NO by measuring NO metabolites. However, chemiluminescence is a direct method for NO measurement in the gas phase. It is based on the interaction of ozone and NO that generates light. A sample containing gaseous NO is mixed with ozone and the emitting light is measured by a photodetector. The amount of light is proportional to NO levels in the sample [122].

Increased NO formation has been shown in numerous inflammatory conditions such as asthma, colitis, cystitis, rheumatoid arthritis and pelvic inflammatory disease [123-130]. In patients with asthma, NO levels in orally exhaled air have shown a 2-10 fold increase compared to healthy controls and the levels correlate with disease severity. Glucocorticoid treatment of inflammation are known to inhibit iNOS and treatment with inhaled or oral glucocorticoids reduces NO levels in asthma patients in a dose-dependent way [131, 132]. Using NO values to prescribe a patient's daily dose of inhaled corticosteroids enabled reduction in the total dose of corticosteroid required, compared with traditional spirometry and symptom based approaches and has been suggested as a quick and non-interventional method with high patient acceptability [124, 133]. In patients with interstitial cystitis there is a significant correlation between symptoms and changes in NO concentration [134]. This implies that NO measurement can be used for diagnosing and monitoring the grade of inflammation in affected organs and to evaluate the treatment response in patients with various inflammatory diseases [124, 134]

However, NO may cause local and systemic tissue damage and thereby contribute to the morbidity of infections. The exact role *in vivo* is yet to be clarified and the beneficial and harmful effects of NO need further elucidation.

1.2.2.6 Antimicrobial peptides

Antimicrobial peptides are important effector molecules of the innate immune system and are present in all kinds of living organisms. They are defined as peptides that contain fewer than 100 amino acids and exhibit broad-spectrum antimicrobial activity against bacteria, fungi and enveloped viruses. Nearly all AMPs are cationic and hydrophobic and exert their antimicrobial action by disrupting bacterial, fungal or viral membranes [135]. By electrostatic interaction between the negatively charged microbial membranes and the cationic peptides with hydrophobic properties, the peptide is inserted to the membrane leading to disruption and lysis of the microbe [136-138]. AMPs interact with both the innate and the adaptive

immune systems [139, 140] and can interfere with adhesion of microorganisms and with biofilm formation [141, 142]. In fungus, the membrane is electronically neutral and AMPs seem to target the polysaccharides and surface receptors in the fungal cell wall [143, 144]. Against viruses the AMPs use a wide variety of antimicrobial activities. In enveloped viruses, such as HSV, AMPs target the surface antigen that mediate attachment and fusion with host cells. In the non-enveloped viruses, such as HPV, the target of AMPs is the viral cell entry, un-coating or replication processes [145, 146].

Several families of AMPs have been identified in humans. Major groups are the defensins, cathelicidin and S100 proteins. Defensins are 2- to 6-kDa peptides with six cysteines and three disulfide bridges. The size and the organization of the disulfide bridges further divides the defensins into two major subgroups: α -defensins and β -defensins. The α -defensins are present in phagocytic cells such as neutrophils and in the paneth cells of the small intestine [147, 148]. They are stored in the granules of neutrophils and contribute to the elimination of ingested microbes [149]. The α -defensin 5 (HD5) is present in the FRT and has been shown to have a potent antiviral activity especially against HPV infections [150]. However, HD5 seems to be almost absent in the TZ of women with cervical HSIL [151]. The β -defensins are present in epithelial and phagocytic cells and are expressed in a variety of tissues including the FRT [152, 153]. Human β -defensin -1 (HBD-1) is constitutively expressed but may also be selectively upregulated whereas HBD -2 -3 and -4 are induced by infectious and inflammatory stimuli [154-156]. LL-37 is the only human cathelicidin and it is expressed in neutrophils and numerous epithelial tissues such as the urinary tract, skin, lung and epididymis [157-161]. Cathelicidin has been demonstrated to have antimicrobial activity against a wide range of pathogens as well as potent chemotactic activity. Moreover, it has been shown to be involved in carcinogenesis and progression in tumors of the breast, ovaries and lungs as well as head and neck squamous cell carcinoma [162]. It is also involved in tissue repair by angiogenesis [163].

The S100 family of proteins consists of several Ca^{2+} binding proteins. Psoriasin (S100A7) is an AMP that belongs to this group. It is a monomeric peptide composed of 101 amino acids, normally existing as a homodimer composed of two monomers [164]. The binding motifs of calcium and zinc are important parts of the peptide and binding of calcium is crucial for the structure development of the protein [165]. It is implicated in several cellular processes such as cell proliferation, apoptosis and differentiation [164]. Psoriasin was initially found in psoriatic lesions and is produced by keratinocytes in skin and other epithelial surfaces [166]. It is present in vaginal fluid and is expressed by epithelial cells in the FRT [144, 167, 168]. The antibacterial activity is dependent on its zinc-binding motif and the action is partly mediated by zinc sequestration since zinc is an essential element in the metabolism of bacteria. Mutation experiments with recombinant psoriasin has confirmed that zinc but not calcium binding is of importance for the antibacterial activity [169-171]. Membrane disruption is the basis of its antimicrobial activity at low pH [172]. Psoriasin protects the skin from colonization and infection by *E. coli* and is thereby an important effector molecule in the cutaneous barrier. In addition, it has been shown that psoriasin expression increases in wound exudate after skin barrier disruption [173]. In higher doses, psoriasin also has bactericidal effect against *Pseudomonas aeruginosa* and *Staphylococcus aureus* [166]. Cystein-reduced psoriasin, but not the oxidized form, is an effective broad-spectrum

fungicide for numerous dermatophytes and *Aspergillus fumigatus*. However, direct activity towards *C. albicans* has not been shown [174].

The expression of psoriasin is induced in various hyperproliferative and inflammatory skin diseases such as atopic dermatitis, mycosis fungoides, Darier disease, lichen sclerosus, actinic keratosis and acne inversa and is considered an important effector of the human dermis [164, 166].

Several AMPs such as calprotectin, lysozyme, lactoferrin, secretory leukoprotease inhibitor (SLPI), human neutrophil peptides 1-3 (HNP1-3) and HBD 1-2 have been detected in vaginal secretions and are produced by the epithelial cells of the FRT [175, 176]. Their importance in specific infections is not clarified, however, the cervical mucus plug has been shown to exhibit antimicrobial activity against GBS and *E coli*, but no antifungal activity against *C. albicans* [177]. In one study, women with BV had significantly reduced concentrations of AMP in vaginal fluid compared to healthy controls and women with VVC. After adequate treatment, the AMP levels in women with BV increased to the levels detected in healthy women [178]. The fact that BV is associated with local deficiency of innate immunity may result in a state of local immunosuppression that could be the reason for the susceptibility to HIV and other sexually transmitted diseases. The higher induction of AMPs in candidiasis is considered to be a result of the proinflammatory tissue response during yeast infections [178].

1.2.3 Adaptive immune defense

The adaptive immune response is activated when the physical barriers have been penetrated and the innate immune response has failed to defeat the invading pathogen. Adaptive immunity is characterized by identification of specific antigen determinants of pathogens and the ability to distinguish one microorganism from another. B-lymphocytes produce specific antibodies, while T-lymphocytes have multiple functions such as to assist in antibody production and participate in the cell mediated defense [179]. Ultimately this response is efficient and leads to direct elimination of the pathogen in most cases. The adaptive system poses an immunological memory which allows a quicker response to a specific antigen when the organism faces the same pathogen in the future.

1.2.4 Inflammation and carcinogenesis

Inflammation is an unspecific defense mechanism activated by tissue damage. It is estimated that approximately 15% of worldwide malignancies are related to chronic inflammation. The process is complex involving both the innate and adaptive immune systems. The function of inflammation is to restore the homeostasis by destroying the source of tissue-threat and repair the injury. If the acute disorder is not resolved and instead persists, the inflammation may become chronic which demands constant cell renewal. Thereby the inflammation leads to extended cell division and an increased risk of mutation and malignant cell transformation [180]. Several high-risk HPV genotypes are crucial infections associated with cancer. Other

oncogenic viruses are hepatitis-B virus, Epstein-Barr virus and HTLV-1. Prostaglandins are released from inflammatory cells by the action of the cyclooxygenase enzymes, COX-1 and COX-2, which intensifies the inflammation. The prostaglandin PGE₂ which is produced in many human tumors and promotes malignant growth is induced by COX-2. It is shown that nonsteroidal anti-inflammatory drugs (NSAID) and COX-2 selective inhibitors decrease the risk of several cancer forms like colorectal cancer [181, 182]. HPV infection per se is not enough for malignant transformation of the infected cell. However, the inflammation facilitated by altered immunological mechanisms contribute to the neoplastic process [183-185].

1.2.4.1 Antimicrobial peptides in the neoplastic process

The role of AMPs in carcinogenesis is not yet clarified. Through their involvement in the inflammatory process they can modify the microenvironment by promoting or reducing the development and cancer progression [186].

Human beta defensins presumably contribute to both innate and adaptive immune responses against HPV induced epithelial lesions. Induction of HBD-2 and HBD-3 has been reported in lesions of recurrent respiratory papillomatosis [187], verrucae vulgaris and condyloma acuminata, [188]. In benign vulvovaginal condyloma acuminata there was a significant up-regulation of HBD-1, -2, -3 and psoriasin compared to normal controls [189].

It has been suggested that HBD-1 acts as a tumor suppressor with high expression in precancerous cutaneous lesions and a significant deprivation of HBD-1 expression is seen in renal and prostate cancer [190]. In a study using immunohistochemistry, HBD-1 expression was analyzed in squamous cell carcinomas and showed lower expression compared to premalignant lesions [191]. These findings suggested that the protein expression is decreased during the progress to invasive cancer. In an oral squamous cell carcinoma (OSCC) cell line, HBD-1 acted as a tumor suppressor by inhibiting cell proliferation [192]. In patients with OSCC, HBD-1 was significantly reduced indicating that the lower concentration might contribute to the malignant progression [193]. On the other hand, HBD-2 and HBD-3 increased cell proliferation *in vitro* and were significantly increased together with psoriasin in SCC and SCC-in situ. In anal intraepithelial neoplasia (AIN), HBD-1 expression did not differ from unaffected anal mucosa [194] but the expression of HBD-2 and -3 was significantly increased.

When detecting protein concentrations of HBD-1 in cervico-vaginal lavage from patients with HSIL, the levels were reduced compared to controls. The same study showed significantly lower concentrations of HBD-2 and -3 which is surprising since they are typically up-regulated in response to inflammation and infection [195]. Moreover, it has been discussed if polymorphism in DEFB1, the gene coding for HBD-1, is associated with the ability to clear HPV infection. In a population of Brazilian women two different polymorphisms were associated with susceptibility to HPV infection [196].

The cathelicidin LL-37 is overexpressed in ovarian, breast and lung cancer and it has been shown that LL 37 can contribute to tumor progression and metastasis [186]. However, LL-37 has also been shown to have antitumor effect by inducing apoptosis.

Psoriasin is considered to have the potential to promote tumor progression through various signaling pathways [164]. It is involved in keratinocyte differentiation and enhances the expression of several differentiation markers. Furthermore, psoriasin stimulates endothelial cell proliferation by enhancing vascular endothelial growth factor (VEGF) which is an important growth factor in angiogenesis in both chronic inflammation and cancer [197]. In several tumors, such as squamous cell carcinoma, breast and bladder cancer, psoriasin is overexpressed [191, 198-201]. In estrogen receptor-negative invasive breast carcinoma the expression of psoriasin correlates with tumor progression and acquisition of metastatic phenotype and is associated with a poor prognosis [202, 203]. Since psoriasin seems to be a contributing factor in local tumor progression it might be a valuable diagnostic marker for early diagnosis of primary and recurrent squamous cell carcinoma [204, 205].

2 AIMS

The aims of this thesis were to improve the understanding of the innate immune response in two common infections of the lower female genital tract, recurrent vulvovaginal candida infection (RVVC) and HPV induced high grade squamous intraepithelial lesions (HSIL) of the cervix.

The specific aims of the studies were:

- To investigate the vaginal NO levels and the expression of iNOS in vaginal biopsies before and after treatment of an acute RVVC episode compared to healthy controls and to correlate NO levels with symptoms and clinical findings.
- To investigate the interaction between *C. albicans* and epithelial defense mechanisms, in particular antimicrobial peptides, during RVVC and possible mode of action of psoriasin in the mucosal immunity against *C. albicans*.
- To study the expression of antimicrobial peptides and the proinflammatory cytokine IL-8 in HPV induced cervical highgrade squamous intraepithelial lesions before and after surgical excision compared to controls.
- To study the effect of chlorhexidine digluconate alone and in combination with fluconazole on *C. albicans* eradication and biofilm in RVVC strains.

3 PARTICIPANTS

3.1 SUBJECTS AND ETHICS

Two groups of patients and three groups of controls were recruited for the studies. In Study I and 2 patients with RVVC and healthy women with no history of RVVC were included. The inclusion groups in Study III were patients with HSIL and healthy women without HSIL or HPV infection. In Study IV, candida strains from patients with RVVC were included together with commensal isolates from asymptomatic women. The studies were approved by the regional ethics committee in Stockholm. All participants received oral information and signed consent was obtained from all participants.

3.1.1 Patients with RVVC

Fifty-eight women with a history of RVVC were invited for a recruitment visit at the Vulvar out-patient Clinic at Danderyd Hospital, Stockholm, Sweden, during an acute episode of vulvovaginal candidiasis. Inclusion criteria were age 18-40 years, 3-4 self-reported candida infections the last year, symptoms of an acute candida infection and positive culture for *C. albicans*. All patients presented typical symptoms of acute vulvovaginal candida infection at the recruitment appointment. Exclusion criteria were severe illness, pregnancy, and other genital infections such as *Chlamydia trachomatis*, bacterial vaginosis and other fungal species than *C. albicans* in cultures. Additional exclusion criteria for all participants were vaginal treatments of any kind, vaginal intercourse in the last 24 hours, vaginal douching and ongoing menstruation as well as current antibiotic treatment or other medications that may influence the immune system. Of these, twenty-eight patients fulfilled the inclusion criteria's and were included in the study (mean age 30 years). None of the participants had used antifungal therapy at the time of the first sampling. Patients came for a second study visit after completing treatment with oral fluconazole 50 mg per day for one week followed by oral fluconazole 150 mg a week for five consecutive weeks. At the second visit, the same protocol was used. Recruitment and inclusion flow-chart is shown in *Figure 7*. Sixteen of the same participants were included in Study II (mean age 32 years). *C. albicans* isolates from 18 of the RVVC patients (mean age 30) were included in Study IV.

3.1.2 Healthy control women with no history of RVVC

Forty-seven healthy women with no history of RVVC were invited as potential control subjects. They were recruited via advertisement at Karolinska Institutet and hospitals in Stockholm. Thirty-one women (mean age 26 years) were included in Study I and twenty-seven women (mean age 26) were included in Study II. The exclusion criteria were the same as for patients. The controls had a one single appointment and had not used anti-fungal

treatment before the examination. In Study IV, 19 commensal strains from asymptomatic women were used as controls.

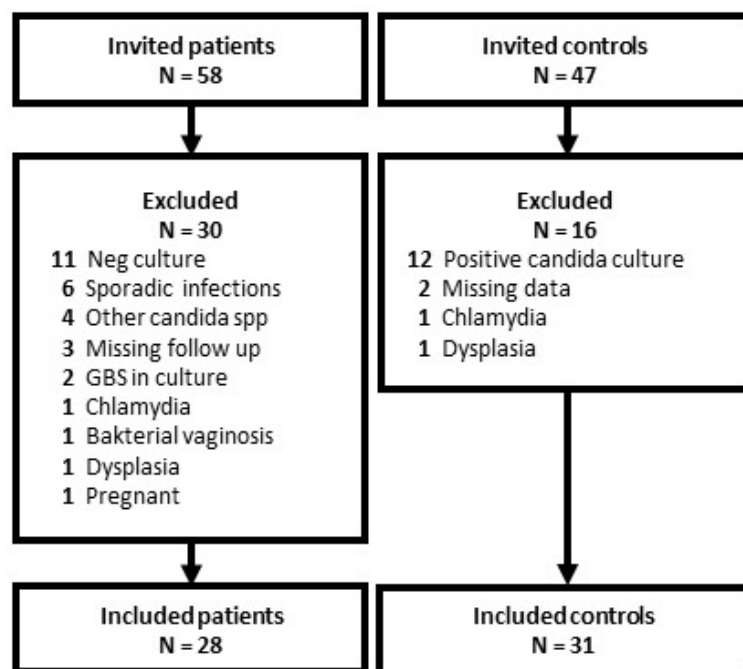


Figure 7. Flow-chart showing the recruitment procedure of patients with RVVC and healthy controls to Study I.

3.1.3 Patients with HSIL

Women referred to the gynecology outpatient clinic at Danderyd Hospital, Stockholm from the National cervical screening program with smears results indicating HSIL were invited to participate. The inclusion criteria were age 18-40 years and diagnosis of CIN 3 verified in surgical specimens after cervical conisation by two independent pathologists. Exclusion criteria were the same as in Study I, II and IV.

After vaginal cultures and histo-pathological analyses were completed, nineteen women (mean age 30 years) with HSIL were included in Study III, *Figure 8*.

All patients were treated with cervical conisation (laser or loop excision) and came back for a second visit 6 months after surgery. All examinations and samplings were repeated at the follow up, according to the same protocol as during the first visit.

3.1.4 Healthy controls with no HSIL

Fourteen women (mean age 31 years) with no history of LSIL or HSIL were enrolled as healthy controls. The exclusion criteria were identical as for the controls in Study I, II and IV.

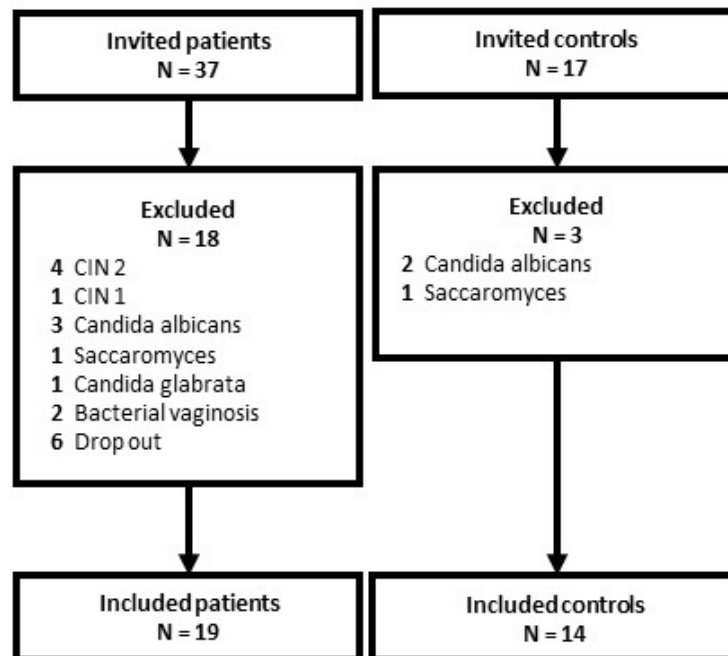


Figure 8. Flow-chart showing the recruitment procedure of patients with HSIL and healthy controls to Study III.

4 METHODS

4.1 QUESTIONNAIRES

All participants filled in a study specific questionnaire surveying age, occupation, smoking habits, ongoing medication and contraception use. In addition, a medical history including, gynecological and reproductive history was taken. For current symptoms of RVVC, a thorough description of discharge, pruritus dryness, soreness and pain was obtained. A symptom score was created, ranging from 0 to 5, based on the number of reported symptoms.

4.2 NO-MEASUREMENT

Measurement of NO levels were achieved prior to the gynecological examination since manipulation of tissue may increase the levels of NO metabolites, likely because of the mechanical provocation [206]. The method of NO measurement in the vagina was described by Sioutas et al [207] and has also been evaluated for use in the uterine cavity by the same group [208]. NO levels were measured by using a small all-silicon catheter. The catheter balloon was positioned in the vagina and filled with 25 ml room air, *Figure 9*. After five minutes' incubation, the air was aspirated into a syringe and directly injected into a chemiluminescence analyzer (CLD 77 AM, Eco Physics) for measurement of NO levels as parts per billion (ppb). The level of NO in the ambient air was measured and subtracted from the NO levels measured in the vagina.

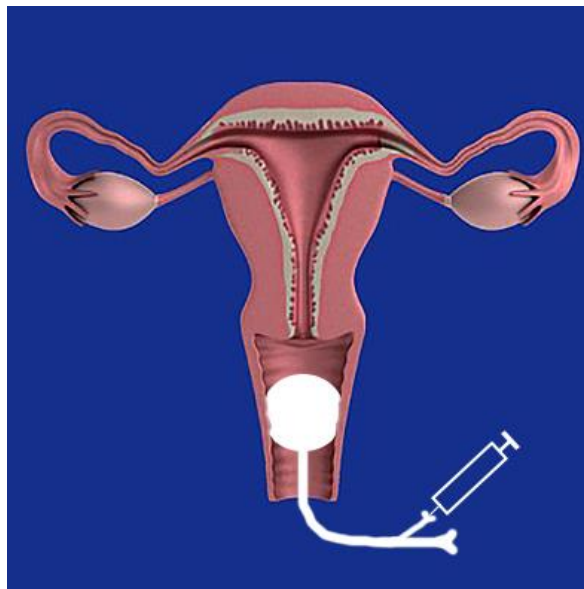


Figure 9. NO measurement in vagina with a catheter balloon.

4.3 EXAMINATION AND SAMPLING

A thorough gynecological examination was made with inspection of the tissues in the vulva and vagina. Microscopy of wet mount with saline and 10% potassium hydroxide (KOH) was performed and specimens for microbiological culture and chlamydia PCR were collected. Amsel's criteria were used for exclusion of bacterial vaginosis (vaginal pH>4.5, positive whiff test, homogenous vaginal discharge and presence of clue cells) [209].

Based on the clinical findings of vaginal erythema, pathological discharge, dry vulvar skin, fissures and visible hyphae in wet mount an examination score was created. Each positive finding generated one point (0-5), Table 3.

In Study II, vaginal lavage was obtained by intravaginal administration of 5 milliliters of distilled water. After 5 minutes the lavage fluid was collected through a small catheter. The fluid was centrifuged at 300xg for 10 minutes and then sterile-filtrated. The cell pellet and the supernatant were stored separately at -80°C.

Sampling of mucus from the cervical canal for Study III, was performed using a cytobrush [210]. The cytobrush was stirred for 1 min in a test tube containing 3 ml of sterile water before centrifugation at 300xg for 10 min. The cell pellet and the supernatant were stored separately at -80°C.

The vaginal biopsies for Study I and II was obtained with 3 mm forceps at the 3 and 9 o'clock positions approximately 3-4 cm from the vaginal introitus. Before obtaining the biopsy, 2 mL of the local anesthetic lidocaine was injected in the tissue. One biopsy was fixed in 4% paraformaldehyde at 4°C overnight, transferred to 70% ethanol and processed for immunohistochemical analysis. The second biopsy was placed in RNA-later RNA Stabilization Reagent (Qiagen) and subjected to RNA isolation and gene expression analysis.

For Study III cervical forceps biopsies of 3 mm were obtained from the cervical transformation zone after colposcopy. One biopsy was sent for histo-pathological analysis. A second biopsy was prepared for RNA analysis and a third biopsy was processed for IHC.

4.4 IDENTIFICATION AND ISOLATION OF *C. ALBICANS*

For identification of *C. albicans*, vaginal swabs were cultured on CHROMagar. Candida and typical colonies were further identified by latex surface antigen agglutination (Bichro-Dubli). Absence of fungal and bacterial growth in the control participants was confirmed according to the same procedure. Isolates were kept frozen at -80°C. For experiments, if not stated otherwise, isolates were cultured on Sabouraud agar for 1-2 days at 37°C and then in yeast peptone dextrose (YPD) medium overnight at 30°C. This culture was diluted 1:100 in fresh medium and grown for another 3 hours at 30°C to the logarithmic growth phase. Under these conditions, *C. albicans* grew as yeast cell.

4.5 IMMUNOHISTOCHEMISTRY

Biopsies collected for immunohistochemical analysis were paraffin-embedded and cut at 4µm and mounted onto glass slides.

For Study I immuno-staining for iNOS was manually performed using EnVision™ Peroxidase/DAB Detection System kit, according to instructions provided by the manufacturer. The specificity of the immune-staining was confirmed by lack of staining after omitting the primary antibody. Staining for NADPH-d or iNOS was performed simultaneously on all sections to avoid variability in staining intensity. A semi-quantification of the general iNOS staining was defined as absent=0, mild=1, moderate=2 or intensive=3 by two independent investigators blinded to the protocol using a Zeiss Axiophot microscope.

For Study II and III the sections were de-paraffinized in xylene or Neo-Clear, rehydrated in an ethanol gradient and washed in water and phosphate-buffered saline (PBS) and thereafter prepared according to an IHC protocol. Overnight incubation with primary antibodies was carried out at 4°C. Primary antibodies used were rabbit anti-HBD1 (1:100, Santa Cruz Biotechnology) (Study II), goat anti-HBD2 (1:50, R&D Systems,),(Study II), rabbit anti-HBD3 (1:100, Santa Cruz Biotechnology)(Study II), rabbit anti-psoriasin (1:800, Abcam,)(Study II and III), and rabbit anti-RNase 7 (1:500, Novus Biologicals)(Study II). Sections were then incubated with Alexa Fluor-conjugated secondary antibodies mounted in medium with DAPI (Life Technologies). Images were acquired on a Leica SP5 (Wetzlar,) confocal microscope using a 40× objective.

4.6 TOTAL RNA EXTRACTION AND REAL-TIME PCR ANALYSIS

Total RNA extraction from biopsies and cultured cells was extracted using the RNase Mini kit (Qiagen). In Study II reverse transcription with up to 1 µg of RNA was carried out using the DyNAmo cDNA Synthesis Kit (Finnzymes,) or the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). In study III the concentration and purity of RNA were determined with Nanodrop, and up to 1 µg of RNA was transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific).

Gene expression was analyzed with TaqMan gene expression assays (Thermo Fisher Scientific Baltics,) for AMPs and cytokines as shown in *Table 1*. Expression of RNase 7 was examined using a SYBR Green-based assay (Qiagen).

Human GAPD (GAPDH, 4326317E) and 18S rRNA was used as housekeeping controls to calculate relative expressions of target genes.

Table 1. Analyzed genes by PCR

AMP/Cytokine	Expressed gene
Human β -defensin 1	DEFB1, Hs00608345_m1
Human β -defensin 2	DEFB4A, Hs00175474_m1
Human β -defensin 3	DEFB103A, Hs00218678_m1
Psoriasin	S100A7, Hs00161488_m1
Human cathelicidin LL-37/ hCAP-18002C	CAMP, Hs00189038_m1
IL-1 β	IL1B, Hs01555410_m1
IL-6	IL6, Hs00985639_m1
IL-8	IL8, Hs00174103_m1

4.7 WESTERN BLOT

For detection of psoriasin in cell pellets, cells were lysed and cleared by centrifugation and the protein concentration was determined by BCA assay. Equal amounts of protein were heated and subjected to polyacrylamide-gel-electrophoresis and the separated samples were transferred to PVDF membranes. After transfer, membranes were blocked and incubated with a polyclonal rabbit anti-psoriasin (1:800 in milk-TBST) antibody. Incubation with an anti-rabbit HRP-conjugated secondary antibody was carried out and signals were detected.

4.8 ENZYME-LINKED IMMUNOSORBENT ASSAYS (ELISA)

In Study II vaginal lavage samples were analyzed using ELISA kits for HBD -1, -2 and 3, psoriasin, LL-37, human ribonuclease (RNase) 7 and IL-8 according to the manufacturer's instructions.

In Study III the supernatant from the cervical secretion samples were analyzed using ELISA kits for psoriasin and IL-8 and were carried out according to the manufacturer's recommendations. The levels of psoriasin and IL8 in each sample were presented as the ratio of the proteins and the total protein content in secretion samples determined by using BCA assay.

4.9 CHEMICALS AND REAGENTS

Psoriasin was purified from human skin [211], dissolved in 0.01% acetic acid to a concentration of 1 mg/ml and stored at -20°C. A vaginal fluid simulant (VFS) was used as medium for all reactions to mimic the vaginal milieu. This previously described formulation resembles the vaginal material with respect to pH, osmolarity and chemical composition

[212]. Organic acids are represented by lactic and acetic acid and proteins by albumin. All mono- and polysaccharides were purchased from Sigma; working dilutions were prepared freshly in VFS.

4.10 CANDIDA ADHESION ASSAY

To determine the adhesive capacity of *C. albicans* isolates, a microtiter plate assay, using VFS as medium or vaginal lavage was used [142]. *C. albicans* yeast cells from the logarithmic growth phase were accumulated by centrifugation and washed twice in PBS. The pellet was dissolved in VFS and the density was adjusted spectrophotometrically. A 100- μ l volume containing approximately 1×10^6 CFU was inserted to wells on 96-well sterile, non-treated, flat-bottomed microtiter plates and incubated for 30 min at 37°C with low agitation (100 rpm). Thereafter, wells were washed with PBS to eliminate non-adherent cells, and the amount of adherent cells were quantified by metabolic activity using a XTT assay. Psoriasin (0.001-1 μ M or vector) was added to duplicate wells to investigate the influence of psoriasin on *C. albicans* adhesion. In selected experiments, psoriasin (1 μ M or vector) was pre-incubated with polysaccharides β -(1,3)-D-glucan, D-mannan and chitin (1 mg/ml in VFS) for 30 min before the addition of *C. albicans*. To evaluate the effect of vaginal lavage on adhesion, VFS was replaced by vaginal lavage fluid from patients and control participants.

4.11 PSORIASIN – CANDIDA BINDING ASSAYS

To demonstrate binding between *C. albicans* cells and psoriasin, a pull-down assay was modified from a previously described protocol [142]. Approximately 1.5×10^7 cells were incubated with 10 μ g psoriasin in VFS (final volume 250 μ l) and incubated for 30 min with agitation; alternatively, 250 μ l of vaginal lavage was used. Cells were collected by centrifugation and washed with VFS. Binding of psoriasin to polysaccharides was investigated in a similar manner. Because of the solubility of mannan, mannan-agarose was used in the test. β -(1,3)-D-glucan from baker's yeast, chitin from crab shells (0.25 mg/ml) or mannan-agarose (30 μ l) were mixed with 10 μ g psoriasin in VFS (final volume 750 μ l) and incubated overnight with agitation. Polysaccharides were then pelleted by centrifugation and washed with VFS. The final pellets were heated in tricine sample buffer and Western blot was performed.

4.12 CELL CULTURE AND CELL INFECTION

The vaginal epithelial cell line AO was used as model of the vaginal epithelium. Cells were cultured in EpiLife Medium with 60 μ M calcium and supplemented with Human Keratinocyte Growth Supplement in a humidified incubator at 37°C with 5% CO₂. *C. albicans* from logarithmic growth were collected by centrifugation, washed in PBS and

aliquots of 3×10^7 cells were transferred in low-binding 1.5-ml tubes. Cells were collected by centrifugation and the pellet was suspended in 98 μ l VFS with 2 μ l psoriasin (1 mg/ml) or vector. The supernatant was discarded and the pellet suspended in 200 μ l 0.2% paraformaldehyd (PFA) in PBS. Cells were suspended in cell culture medium to a final concentration of 10^7 CFU/ml. From this suspension, 100 μ l was added to a well of confluent AO cells containing 900 μ l of fresh cell culture medium and incubated for 6 hours in a humidified incubator. The medium was removed and cells were collected for RNA extraction and gene expression analysis. All conditions were analyzed in duplicate.

4.13 TRANSMISSION ELECTRON MICROSCOPY

Transmission electron microscopy of *C. albicans* incubated with psoriasin or vector (control) for 30 min at 4°C was performed. β -glucan was marked with immunogold-labelled anti- β -glucan antibodies. The cell wall thickness from non-treated and psoriasin-exposed cells in 5-8 images per experiment was measured, three independent experiments were performed.

4.14 MICROTITER METHOD TO MEASURE *C. ALBICANS* BIOFILM

To measure the ability of *C. albicans* to adhere and form biofilm, the crystal violet assay in polystyrene microtiter plates was performed. *C. albicans* were grown for 48h in YPD at 30°C without shaking. Biofilm was stained with crystal violet (0.3%). The dye was solubilized with 80% ethanol and 20% acetone and the optical density was measured at 570 nm.

4.15 CRYSTAL VIOLET METHOD TO ANALYZE THE EFFECT OF CHLORHEXIDINE DIGLUCONATE AND FLUCONAZOLE ON BIOFILM AND *C. ALBICANS*

The effect of chlorhexidine digluconate (0.02%, GlaxoSmithKline) and fluconazole (4 μ g/ml, F8929, Sigma) was evaluated on biofilm and growth of *C. albicans*. The effect of chlorhexidine digluconate on formation of biofilm were analyzed using the crystal violet method. Each candida strain, in concentration 5×10^6 , was treated with 0.02% of chlorhexidine digluconate for 48 h, in 30°C. Planktonic cells were removed, wells were washed twice with PBS and then stained for 10 minutes with 0.3% crystal violet. After removing crystal violet and washing, a solution of acetone and ethanol was used to dissolve the remaining crystal violet stains from the biofilm, the OD was analyzed using spectrophotometer at 570 nm.

To investigate the effect on mature biofilm on the other hand, formation was allowed for 48 hours, before chlorhexidine digluconate 0.02% or fluconazole 4 μ g/ml or a combination of chlorhexidine digluconate and fluconazole were added for 24 h. The optical density of the

chlorhexidine digluconate treated group with or without fluconazole was compared with an untreated control group.

4.16 ENUMERATION OF *C. ALBICANS* IN MATURE BIOFILM

C. albicans were enumerated, following biofilm formation and exposure to chlorhexidine digluconate, fluconazole and the combination of chlorhexidine digluconate and fluconazole or medium alone. To investigate if the tested drugs had impact on the mode of candida growth, the number of planktonic cells as well as cells within the biofilm were analyzed. Planktonic candida cells detected in the medium were estimated by viable count after serial dilutions in PBS. To assess the number of candida in the already established, mature, biofilm, the biofilm was first washed twice with PBS and then scraped from the microtiter plate. The biofilm was dissolved in 200 µl PBS, and serially diluted before viable count was performed. Dilutions were plated on blood agar plates followed by overnight incubation in 37°C and results from cells exposed to test drugs or medium alone were calculated.

4.17 STATISTICS

The IBM SPSS Statistics for Windows (Version 22, 0,) and GraphPad Prism, version 5 and 6 (GraphPad Software) were used to analyze the data. For comparison of clinical characteristics between patients and controls, either Student T-test or Fischer's exact test were used as appropriate. Data obtained from clinical materials were analyzed with non-parametric tests, results presented as individual values with median. For matched comparisons, the Wilcoxon signed rank test or Friedman test was used, whereas comparison between groups was performed by Mann Whitney or Kruskal-Wallis tests. For correlating numeric and categorical variables such as vaginal NO levels to the symptom and examination scores, the Spearman's rank correlation test was used and Pearson correlation coefficient for comparisons of numeric variables, such as mRNA and protein levels of psoriasin and IL-8. Multiple comparisons of AMPs in Study II were analyzed by unpaired t-test, one-way ANOVA and Dunnett's multiple comparison tests, or two-way ANOVA and Bonferroni's multiple comparison, which was also used for analyzing the formation of treated and untreated biofilm and cell growth by viable count in Study IV. Data from other in vitro experiments in Study II are presented with mean and standard deviation from at least three independent experiments performed in at least duplicate. Variations in the number of participants included were due to limited material and biological outliers were not excluded, whereas technical outliers were detected by Grubb's test and excluded. All tests were performed two-sided and differences with P-values of <0.05 were considered statistically significant.

5 RESULTS

5.1 STUDY I: NITRIC OXIDE IN RECURRENT VULVOVAGINAL CANDIDIASIS

5.1.1 Clinical background

The clinical background of patients and healthy controls in Study I are shown in *Table 2*.

Table 2. Clinical data of participants

Clinical variable	Patients, n=19 Mean/No (range/%)	Controls, n=14 Mean/No (range/%)	P-value
Age	30 (23-40)	31 (23-39)	ns
BMI	23.0 (18.8-28.3)	23.8 (20.0-32.3)	ns
Pregnancies	1.5 (0-6)	1.9 (0-5)	ns
Parity	0.8 (0-4)	1.6 (0-4)	ns
Smoking	4 (21)	2 (14)	ns
Contraceptives:			
Combined	6 (32)	0	ns
Gestagen	5 (26)	4 (29)	ns
Cu- IUD	1 (5)	0	ns
Condom	0	1 (7)	ns
History of infections:			
Chlamydia	6 (32)	3 (21)	ns
Herpes	1 (5)	0	ns
BV	1 (5)	2 (14)	ns
Condyloma	3 (16)	0	ns
Candida	8 (42)	7 (50)	ns
Previous Dysplasia	3 (16)	0	ns

The patients were older and reported significantly higher numbers of previous condyloma and BV. However, they did not have a history of more frequent other genital infections or cervical dysplasia compared to the healthy controls. No other differences were detected between the groups.

5.1.2 Symptoms and clinical findings

When patients came for the recruitment visit they reported symptoms associated with vulvovaginal candida infection. Most patients presented with several symptoms and clinical findings of VVC. According to the inclusion criteria, all patients had *C. albicans* in culture. At the follow up visit after treatment, the symptom and examination scores were significantly lower ($p < 0.001$), *Table 3*. However, four patients had positive culture and six patients had visible hyphae in wet-mount. One of the patients had symptoms of pruritus and both positive culture and visible hyphae. Five controls with no clinical signs of candida infection had hyphae in wet-mount.

Table 3. Symptoms and clinical findings

Variables	Patients before treatment n= 28 (%)	Patients after treatment n=28 (%)	Controls n=31 (%)
Symptoms			
Discharge	24 (86)	8 (29)	0 (0)
Pruritus	21 (75)	6 (21)	0 (0)
Dryness	21 (75)	14 (50)	0 (0)
Soreness	26 (93)	9 (32)	0 (0)
Pain	21 (75)	10 (36)	0 (0)
Symptom score 0-5 mean (median)	4.04 (4) ^{a, b}	1.61 (1)	0 (0)
Clinical findings			
Vaginal erythema	26 (93)	3 (11)	1 (3)
Pathological discharge	21 (75)	2 (7)	1 (3)
Dry vulvar skin	18 (64)	6 (21)	1 (3)
Fissures	6 (21)	2 (7)	0 (0)
Hyphae in wet-mount	27 (96)	6 (21)	5 (16)
Positive <i>Candida albicans</i> culture	28 (100)	4 (14)	0 (0)
Examination score 0-5 mean (median)	3.54 (4) ^{c, d}	0.68 (0)	0.26 (0)

Comparisons: ^{a + c)} patients before and after treatment $p < 0.001$, ^{b + d)} patients before treatment and controls $p < 0.001$.

5.1.3 NO levels in patients and controls

Patients with acute infection of VVC displayed median vaginal NO levels of 352 ppb (range 0-6178) compared to a median of 6 ppb (range 0-43) in healthy controls, $p < 0.001$, *Figure 10a*. When fluconazole treatment for six weeks were completed, the NO levels in the patients were reduced to a median of 18 ppb (range 0-163), $p < 0.001$, *Figure 10b*. The levels were however significantly higher compared to the controls, $p = 0.003$. After treatment, four of the patients displayed positive candida cultures, but did not have symptoms of infection. A post priori analysis was performed, including only patients with negative cultures, showing that the NO levels were yet higher in patients after treatment, median (22 ppb, range 0-131), compared to healthy controls (median 6 ppb, range 0-43), $p < 0.005$. The method of NO measurement in vagina was well accepted and there were no reports of distress during the testing procedure. In one patient and two controls the measured NO levels were lower than the concentration in ambient air. In those cases, the values were considered outliers and were set to 0 for statistical purposes.

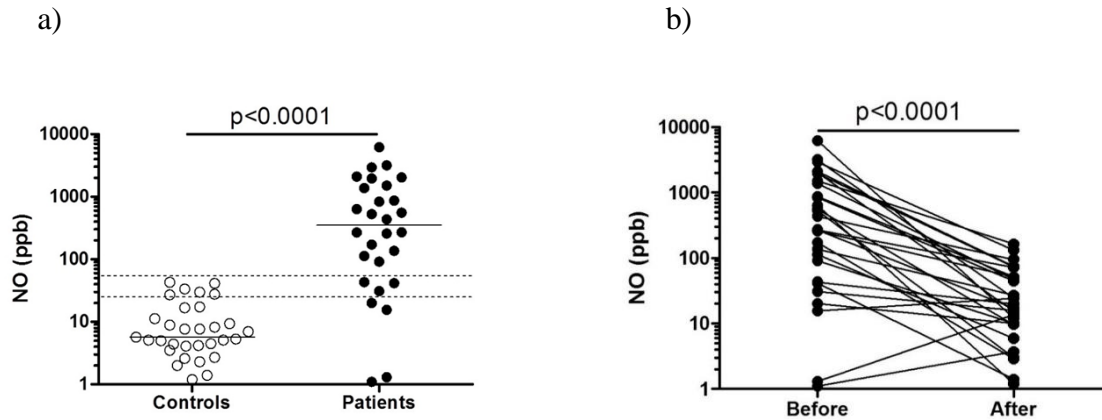


Figure 10. a) Median levels of vaginal nitric oxide in healthy controls and in women with RVVC during an acute exacerbation (ppb=parts per billion). The scale is logarithmic, the dotted lines represent 25 and 50 ppb, b) Vaginal NO levels in women with RVVC before and after six weeks of treatment with fluconazole (ppb=parts per billion).

To evaluate vaginal NO measurements as a diagnostic tool and the examination score for predicting true clinical cases ROC analyses were performed, *Figure 11*. Calculations of sensitivity and specificity for various cut-off levels for NO were used to estimate the effectiveness of NO as a diagnostic tool for vulvovaginal candidiasis. The sensitivity was 0.75 for the cut-off level of NO 50 ppb and specificity 1.0. For NO 25 ppb, the sensitivity was 0.86 and specificity 0.81, *Figure 10a*.

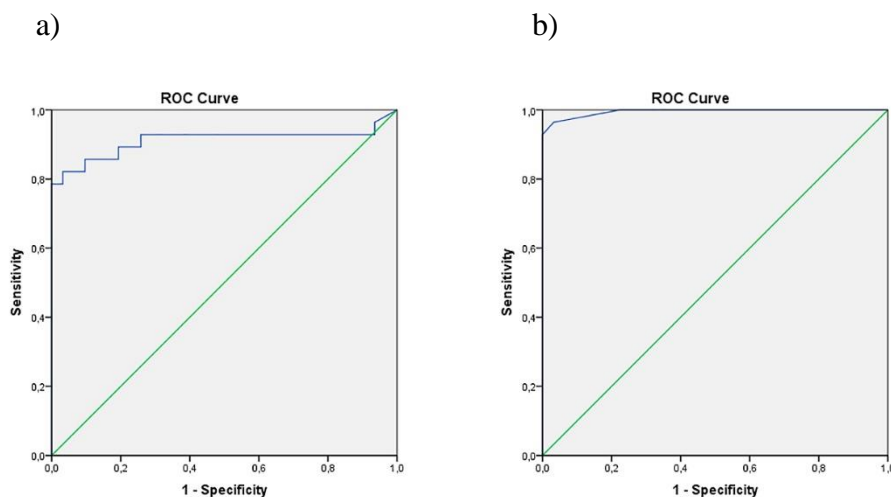


Figure 11. Roc analyses. Twenty-eight patients with acute vulvovaginal candida infection and 31 healthy controls. a) NO measurement, area under the curve 0.911, $p<0.001$ b) Examination score, area under the curve 0.995, $p<0.001$.

5.1.4 iNOS expression

The expression of iNOS was investigated in patients before and after antifungal therapy compared to healthy controls. A difference of the expression within the epithelium was detected. Distinct iNOS staining in the basal cell layers of the epithelium was significantly more common in patients during acute infection (70%) than in controls (20%), $p=0.034$, *Figure 12 a-b*. After treatment, the staining in the basal cell layers was still present more often in patients (67%) than in controls (20%), $p=0.022$. When analyzing the overall iNOS staining in the epithelium, no differences were seen between patients before and after treatment compared to controls. No correlations were found between NO levels and iNOS staining.

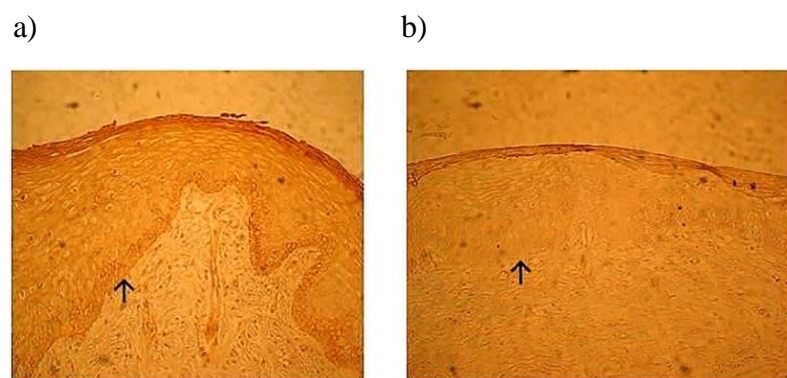


Figure 12. a) Patient with iNOS immunostaining of the epithelial lining and a distinct iNOS expression at the basal membrane layer, b) Healthy control with iNOS immunostaining of the epithelial lining, without a distinct iNOS expression at the basal membrane layer.

The results before treatment show a positive correlation between vaginal NO levels and examination score, $r_s = 0.677$, $p<0.001$, and symptom score, $r_s = 0.644$, $p<0.001$. After treatment when the NO levels were reduced, no correlations to the examination score, $r_s = 0.083$ (ns) or symptom score, $r_s = 0.236$ (ns) persisted.

5.2 STUDY II: PSORIASIN, A NOVEL ANTI-CANDIDA ALBICANS ADHESIN

5.2.1 Clinical background

Six-teen patients with RVVC and 27 healthy controls were included in the study. The controls were younger than the patients, $p=0.04$. All patients had verified *C. albicans* at the first visit, but were culture negative at follow up. There was no difference in contraceptive use between patients and healthy controls.

5.2.2 Expression of antimicrobials proteins and peptides in the vaginal epithelium.

The expression of six epithelial AMPs were analyzed in vaginal biopsies and vaginal lavage from healthy women ($n=27$). Psoriasin, encoded by the S1007A gene and RNase 7 presented the highest mRNA and protein levels, *Figure 13*.

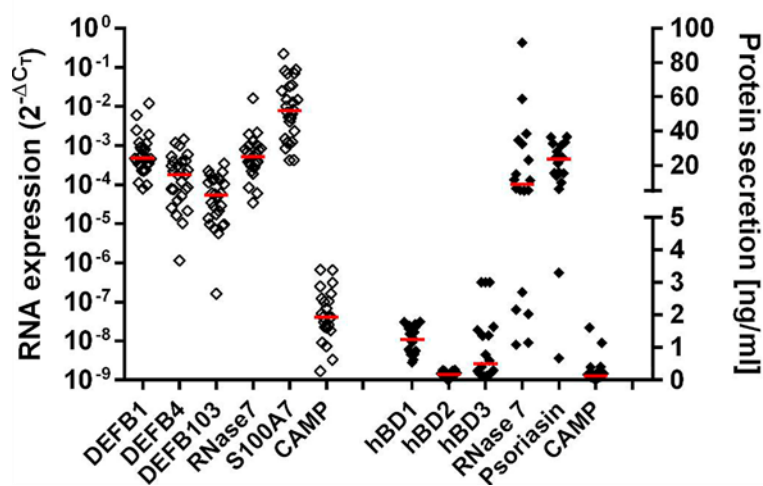


Figure 13. m-RNA expression and protein levels of antimicrobial proteins in vaginal tissue and vaginal lavage in healthy women.

The mRNA and protein levels of the AMPs were analyzed during acute *C. albicans* infection and after completed antifungal treatment. RNase 7 was not increased in response to candida and was not further analyzed. However, psoriasin was strongly up-regulated during the acute infection episode of RVVC, both on mRNA level, *Figure 14a*, and on protein level as shown by immunohistochemistry, *Figure 15*. However, the levels of psoriasin in the supernatant of vaginal lavage was not significantly elevated during infection, *Figure 14b*.

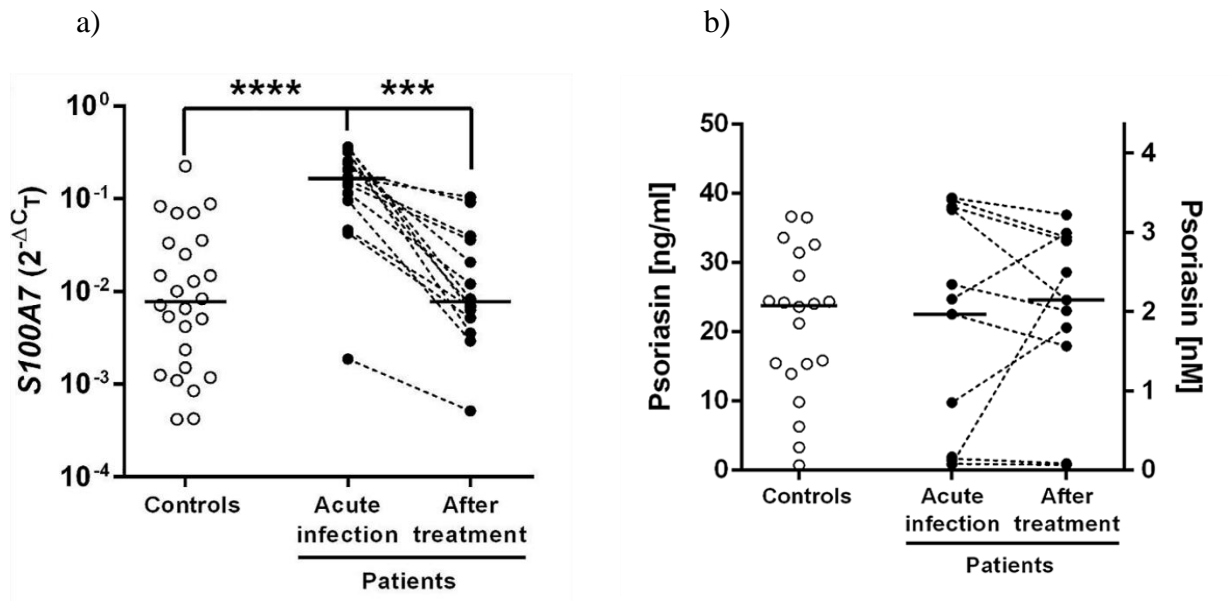


Figure 14. a) Expression of psoriasin, *S100A7*, mRNA in controls and patients during infection and after treatment. b) Psoriasin levels in vaginal lavage samples. *** $P < 0.001$, **** $P < 0.0001$.



Figure 15. Immunohistochemistry in tissue sections, psoriasin: green; cell nuclei: blue.

Increased levels of psoriasin during infection were identified shown by Western blot in the pellet of the vaginal lavage containing desquamated mucosal cells and yeast. Figure 16.

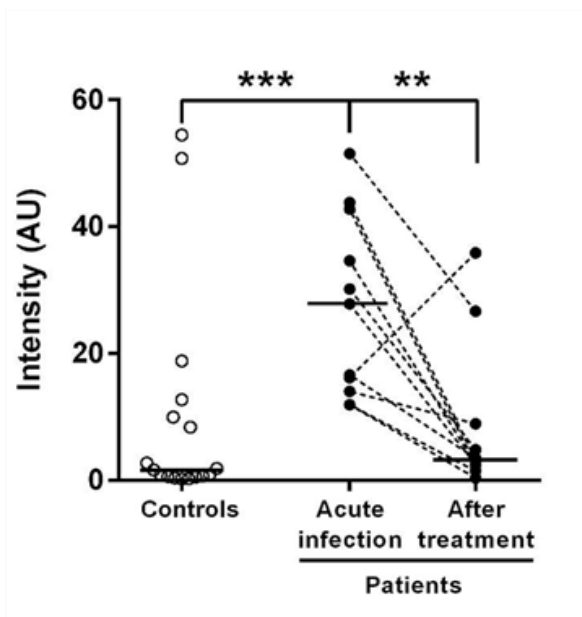


Figure 16. Psoriasin-specific bands on Western blots were quantified, intensity is expressed in arbitrary units (AU)

** $p < 0.01$, *** $p < 0.001$

5.2.3 Psoriasin interacts with β -glucan in the *Candida* cell wall

A possible interaction between psoriasin and the *Candida* cell wall was investigated since psoriasin formed during acute *Candida* infection was associated with the cell pellet, Figure 16. A pull-down assay was performed using *Candida* cells and the polysaccharides β -glucan, chitin and mannan, which are important elements of the fungal cell wall. Both purified psoriasin and psoriasin present in vaginal lavage bound intensely to *Candida* cells and to β -glucan, whereas binding activity by psoriasin to chitin and mannan was weak, Figure 17.

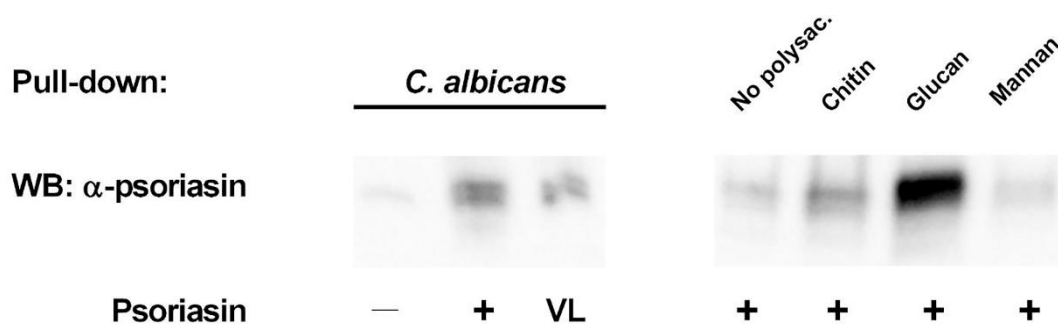


Figure 17. *C. albicans* cells and cell wall polysaccharides were incubated with purified psoriasin or vaginal lavage.

A significant thinning of the cell wall in psoriasin-exposed *C. albicans* was revealed by electron microscopic investigation, $p < 0.05$, Figure 18a–b. Furthermore, reduced content of β -glucan in the psoriasin affected *Candida* cells was shown by immunogold-labelling of the protein, Figure 18a.

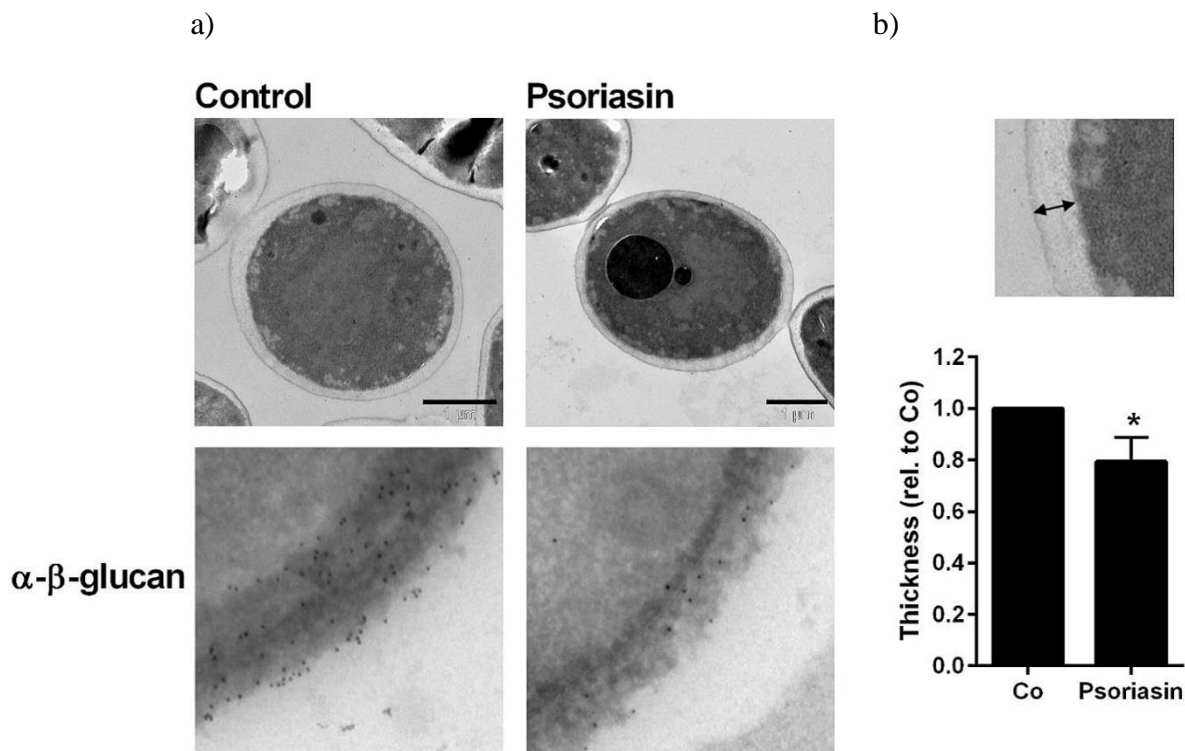


Figure 18. a) *C. albicans* incubated with psoriasin or vector (Control), investigated by transmission electron microscopy, scale bars 1 μ m (upper panel); β -glucan was marked with immunogold-labelled anti- β -glucan antibodies (lower panel, enlarged). b) Thickness of cell wall from non-treated (Co) and psoriasin-exposed cells (Psoriasin), measured as exemplified in the close-up.

5.2.4 Psoriasin inhibits *C. albicans* adhesion to surfaces

The effect of psoriasin-mediated changes of the candida cell wall was analyzed by exploring adhesion of candida in settings simulating the vaginal environment [212]. Psoriasin diminished the adherence of *C. albicans* cells to polystyrene in a concentration-dependent manner, Figure 19a. β -glucan had the capacity to neutralize the anti-adhesive properties of psoriasin, which was not seen with chitin or mannan, Figure 19b.

A similar assay with samples from healthy controls and from patients with acute candida vulvovaginitis was performed to confirm the effect in vaginal lavage. An anti-adhesive activity was shown in vaginal lavage from patients with candida infection and it could, to some extent, be blocked by β -glucan, Figure 19c.

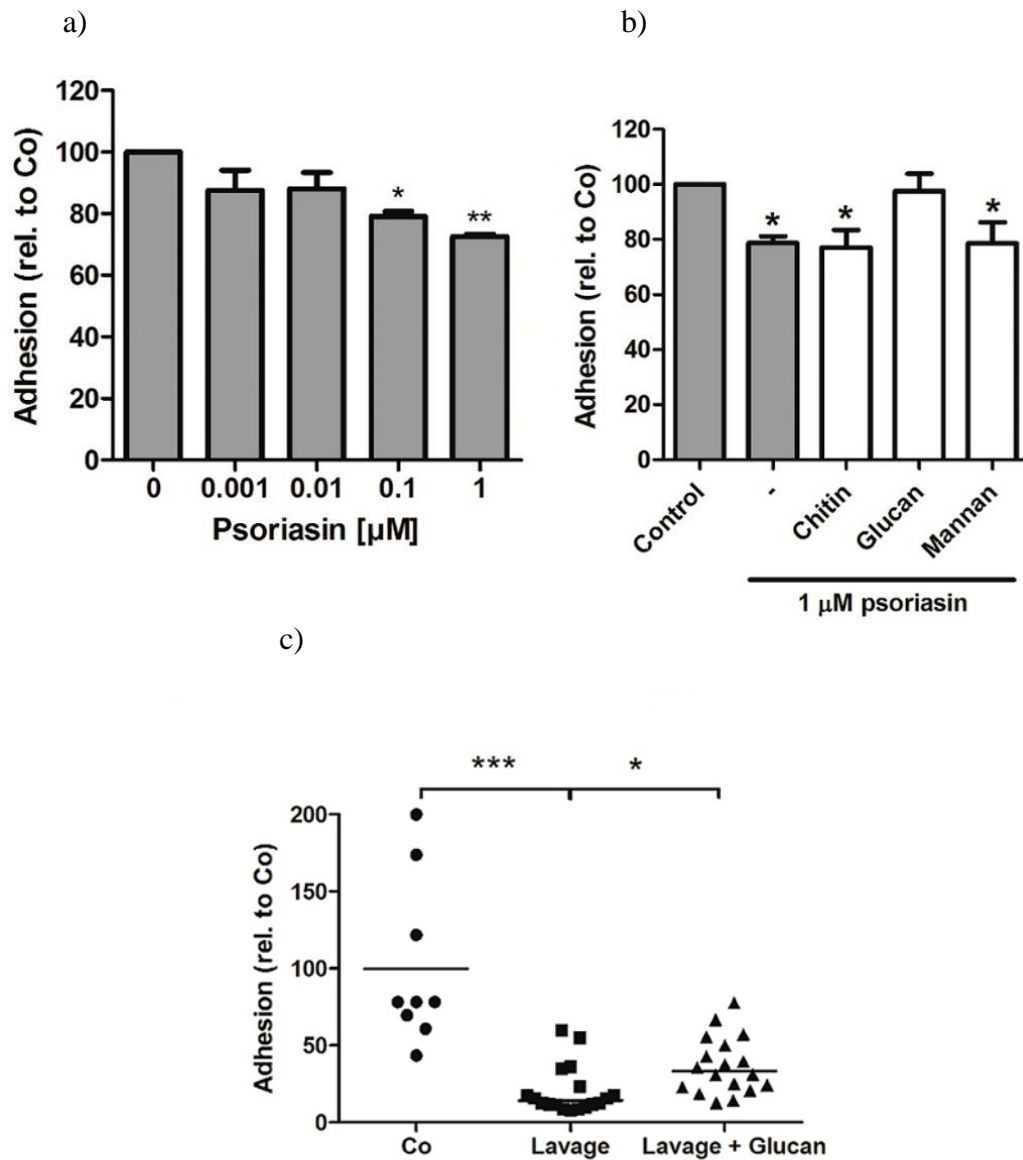


Figure 19. Psoriasin reduces candida adhesion. a) Adhesion of *C. albicans* in vaginal lavage simulant with different concentrations of psoriasin, b) Adhesion of *C. albicans* with psoriasin pre-incubated with polysaccharides. Data are expressed in relation to corresponding samples without psoriasin (Control, Co, =100), c) Candida adhesion analyzed in vaginal lavage samples from healthy controls and patients with recurrent vulvovaginal candidiasis, and in the presence of glucan ($n = 18$).

5.2.5 Epithelial immune response to *C.albicans* is enhanced by psoriasin

Vaginal epithelial cells were infected with psoriasin-treated and non-treated fungal cells to investigate the consequence of psoriasin binding to *C. albicans*. The induction of pro-inflammatory cytokines was influenced by psoriasin treatment of candida cells, the most pronounced effect seen in IL-8 response, *Figure 20a*. Accordingly, IL8 mRNA levels in patients during acute infection showed a positive correlation with levels of psoriasin mRNA, *Figure 20b*. However, psoriasin alone did not influence IL-8 expression in epithelial cells in vitro.

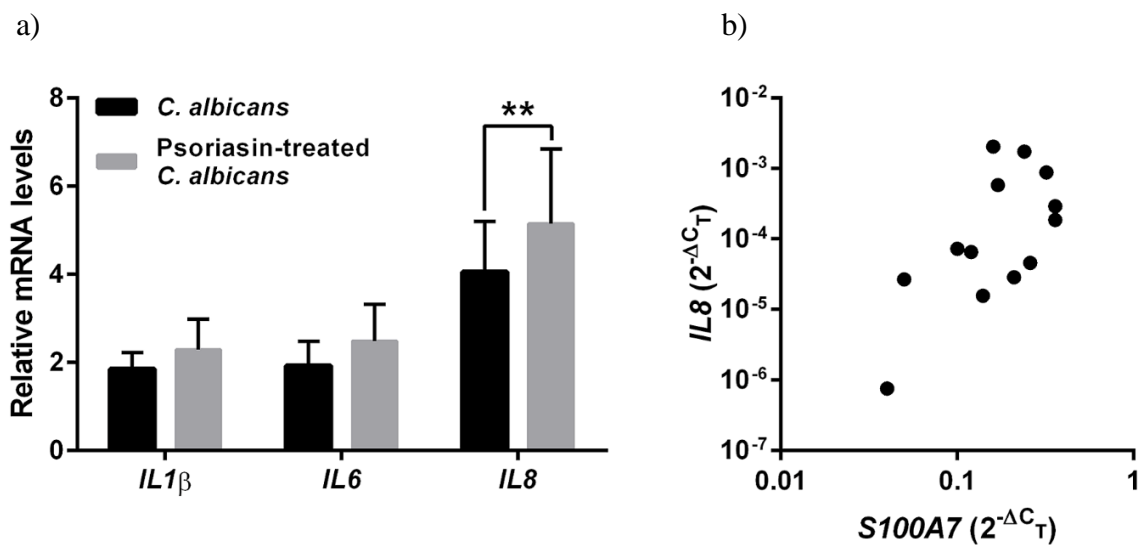


Figure 20 a) mRNA levels of proinflammatory cytokines in vaginal epithelial cells after infection with C. albicans, untreated or pre-treated with psoriasin. b) Correlation between psoriasin mRNA (S100A7) and IL8 expression in vaginal tissue from women during an acute infection with C. albicans, $r=0.55$, $p<0.05$.

5.3 STUDY III: PSORIASIN EXPRESSION IN CERVICAL HSIL

5.3.1 Clinical background

The clinical background of patients and controls are shown in *Table 4*. There were no differences regarding age, BMI, parity, smoking habits or contraceptive use and the history of genital infections was comparable. Three patients described previous low grade cervical dysplasia (LSIL).

Table 4. Clinical data of participants

Clinical variable	Patients, n=19 Mean/No (range/%)	Controls, n=14 Mean/No (range/%)	P-value
Age	30 (23-40)	31 (23-39)	ns
BMI	23.0 (18.8-28.3)	23.8 (20.0-32.3)	ns
Pregnancies	1.5 (0-6)	1.9 (0-5)	ns
Parity	0.8 (0-4)	1.6 (0-4)	ns
Smoking	4 (21)	2 (14)	ns
Contraceptives:			
Combined	6 (32)	0	ns
Gestagen	5 (26)	4 (29)	ns
Cu- IUD	1 (5)	0	ns
Condom	0	1 (7)	ns
History of infections:			
Chlamydia	6 (32)	3 (21)	ns
Herpes	1 (5)	0	ns
BV	1 (5)	2 (14)	ns
Condyloma	3 (16)	0	ns
Candida	8 (42)	7 (50)	ns
Previous Dysplasia	3 (16)	0	ns

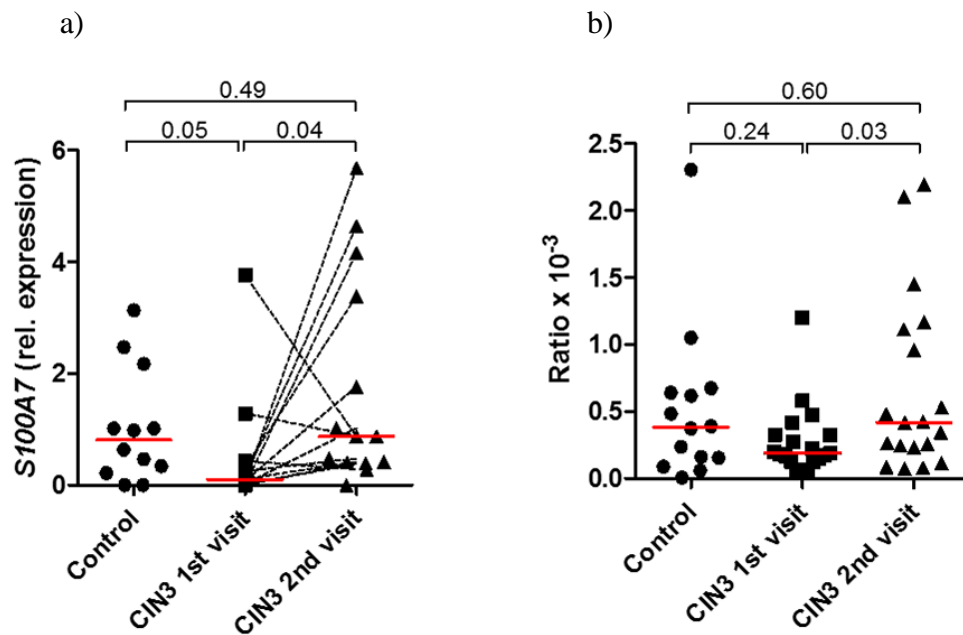
The final inclusion of patients (n=19) was determined by the HSIL diagnosis in the surgical specimen after conisation. At the follow up visit, six months after surgery, three patients had mild atypia in liquid-based cytology. No dysplasia was found in the biopsies of 14 patients and mild dysplasia (LSIL) was diagnosed in one patient who had normal cytology.

Unfortunately, four patients declined the biopsy procedure. Before surgical treatment all patients were positive for HR HPV. At the follow up after conisation, all but two were HPV negative. The HPV positive patients were among the women with mild atypia and subsequently needed further clinical attendance. All controls were HPV negative and had benign cytology and biopsies at the recruitment visit.

5.3.2 The expression of psoriasin

At the first visit before surgical treatment, psoriasin mRNA expression was lower in patients compared to controls, $p=0.05$, *Figure 21a*. Six months' post conisation, the mRNA expression was significantly increased and reached similar levels as in the healthy controls,

$p=0.04$, *Figure 21a*. The protein levels of psoriasin increased significantly after conisation, to levels comparable to those seen in controls, $p=0.03$, *Figure 21b*.



21. Psoriasin in cervical samples from patients with HSIL before and after conisation compared to healthy controls. a) mRNA expression analyzed by real-time PCR b) Protein levels in secretion samples from the cervical canal analyzed by ELISA.

Immunohistochemistry showed that psoriasin is mainly localized in the cytoplasm of the upper epithelial cells. After conisation, the immunostaining was pronounced as opposed to the weak staining of the HPV infected pre-surgical biopsies, *Figure 22 a-c*.

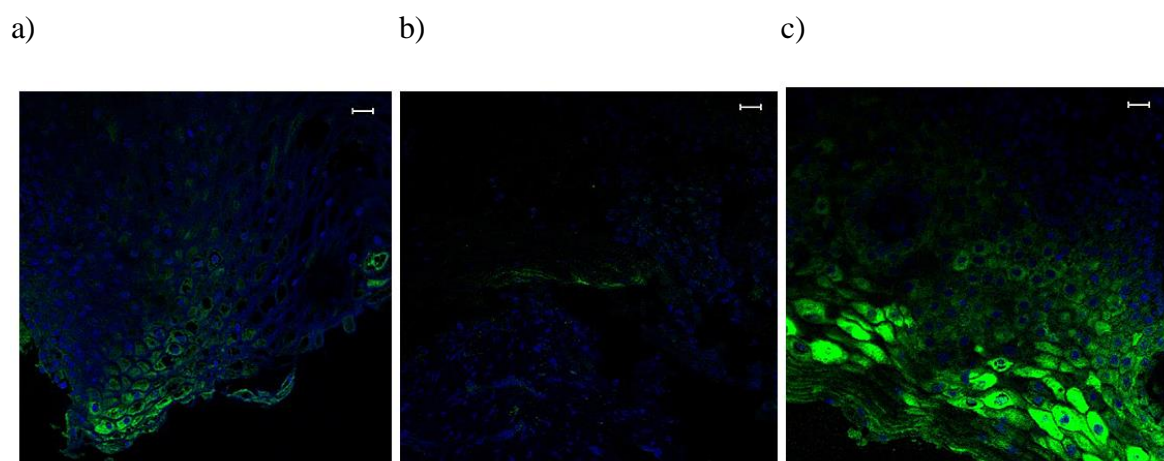


Figure 22. Immunohistochemistry of representative sections (40x objective) from patient with HSIL and healthy control. Epithelial cells with psoriasin staining of the cytoplasm (green) and DAPI to show nuclei (blue). a) Healthy control b) Patient before treatment c) Patient after treatment. Scale bar 20.0 μm.

5.3.3 The expression of IL-8

The proinflammatory cytokine IL-8 was chosen as an indicator of the inflammatory response in cervical tissue. The relative expression of IL-8 mRNA was significantly higher in patients before treatment than six months after conisation, $p=0.05$. No differences were observed compared to controls, *Figure 23a*. On the protein level, no differences were observed in patients before or after treatment nor compared to controls, *Figure 23b*.

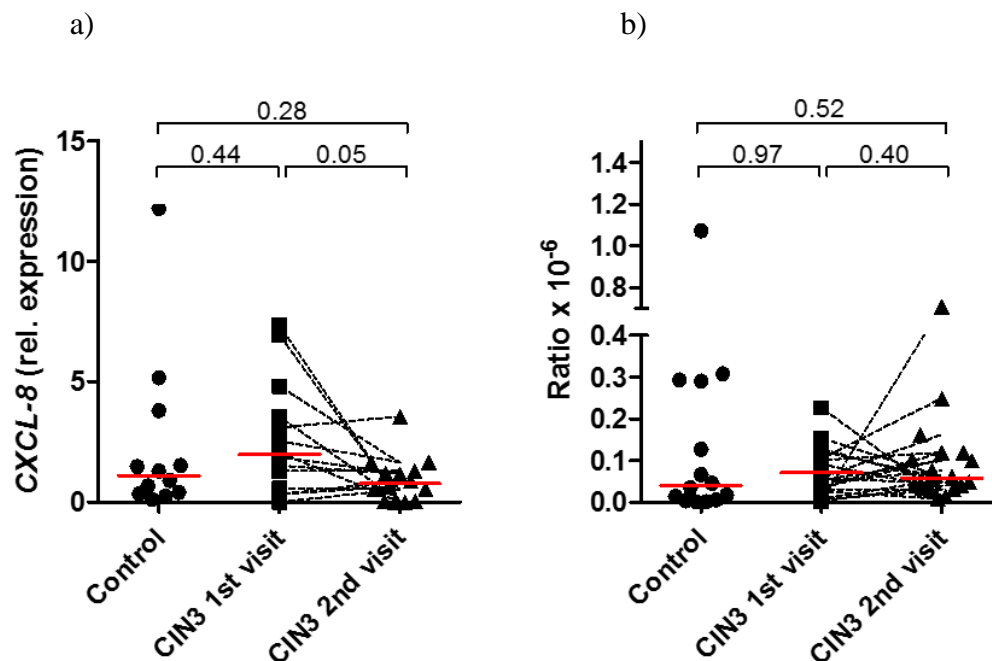


Figure 23. IL-8 in cervical samples from patients with HSIL before and after conisation compared to healthy controls. a) mRNA expression in biopsies analyzed by real-time PCR. b) Protein levels in secretion samples from the cervical canal analyzed by ELISA.

5.4 STUDY IV: NEW TREATMENT STRATEGY OF RVVC

5.4.1 Participants and samples

C. albicans was isolated in vaginal swabs from 18 women, during an acute episode of candida infection and with a history of 3-4 candida infections in the last year. For controls, *C. albicans* isolates from 19 asymptomatic women were collected, referred to as commensals and included for control purposes.

5.4.2 Fluconazole has limited effect on RVVC strains in mature biofilm

Biofilm has an ability to prevent the antimicrobial action of both the innate immunity and antimicrobial treatments. Therefore, the effect of FLZ on *C. albicans* growth in already established biofilm as well as the effect of planktonic cell was investigated, *Figure 24a-c*. There was a significant decrease of planktonic candida cells growing exterior to the biofilm after FLZ treatment ($p < 0.0001$), *Figure 24b*. However, no effect was observed on candida growing within the biofilm, *Figure 24c*. Likewise, FLZ did not affect the biofilm *per se*, *Figure 24a*.

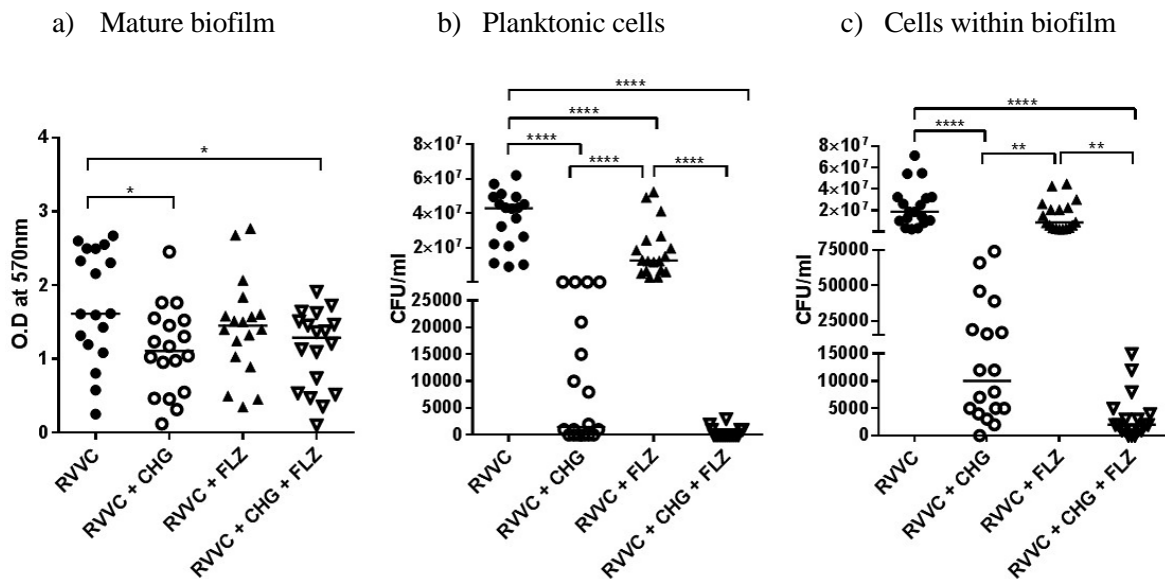


Figure 24. Thickness of established, mature biofilm (a), produced by vaginal *C. albicans* isolates ($n=18$), was measured. The effect of chlorhexidine digluconate (CHG) and fluconazole (FLZ) and the combination CHG + FLZ was investigated. As controls served untreated *C. albicans* from the same patients. Individual and median values are presented, depicted as optical density (OD) at 570 nm after dissolution of crystal violet. Viable count of planktonic *C. albicans* (b) and of *C. albicans* within mature biofilm (c). * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$

5.4.3 Chlorhexidine digluconate dissolves established biofilm of RVVC strains and inhibits candida growth

Since FLZ had a rather poor effect on candida growth and biofilm, an alternative treatment approach was investigated. The effect of CHG treatment alone and in combination with FLZ on *C. albicans* growth and biofilm was analyzed, *Figure 24a-c*. Mature biofilms of RVVC strains were significantly reduced after CHG exposure which was not shown after FLZ treatment, *Figure 24a*. Furthermore, candida cell growth both in planktonic state and within biofilm was significantly reduced by CHG treatment. An additional effect of combined CHG and FLZ was seen, but did not reach statistical significance, *Figure 24b-c*. Moreover, there was no statistical difference observed comparing the effect of CHG alone and in combination with FLZ on established biofilm. However, a sub-analysis of paired samples revealed that all pathogenic strains except two, had lower amount of CFU/ml when FLZ was added as a combined treatment, data not shown.

5.4.4 Biofilm formation can be inhibited by chlorhexidine digluconate in RVVC strains

Based on the effect of CHG on mature biofilm, we sought to investigate if CHG also could inhibit formation of new biofilm and *C. albicans* growth. We observed that 0.02% CHG was able to prevent the formation of biofilm as well as candida growth in planktonic and biofilm state, in RVVC strains, *Figure 25 a-c*.

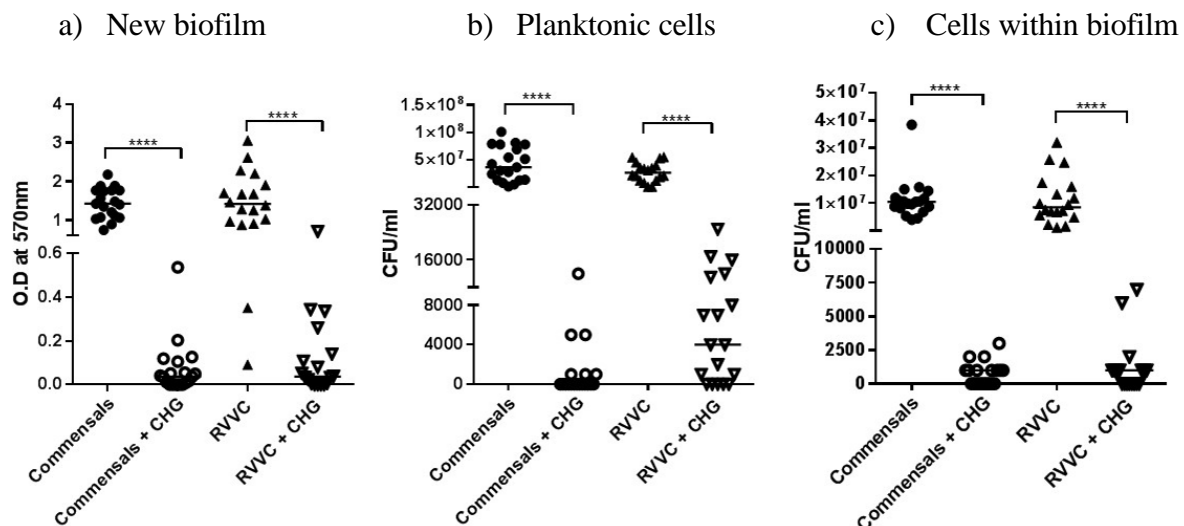


Figure 25. The effect of chlorhexidine digluconate was investigated on formation of new C. albicans biofilm (a). C. albicans were obtained from patients with RVVC (n=18) and from asymptomatic women, commensal strains (n=19). Untreated strains served as controls. Individual and median values are presented, depicted as optical density (OD) at 570 nm after dissolution of crystal violet. Viable count of planktonic C. albicans (b) and of C. albicans in newly formed biofilm (c).

**** $p < 0.0001$

5.4.5 Commensal *C. albicans* growth and biofilm is affected by chlorhexidine digluconate

Commensal *C. albicans* isolates were studied and the capacity to form biofilm did not differ from RVVC strains, *Figure 26a and 24a*. However, contrary to in RVVC strains, CHG as a single treatment and in combination with FLZ did not reduce mature biofilm in commensal strains, *Figure 26a*. Furthermore, in line with the results from RVVC strains, CHG alone and in combination with FLZ was able to inhibit candida growth both in the planktonic phase and within established biofilm, *Figure 26b-c*.

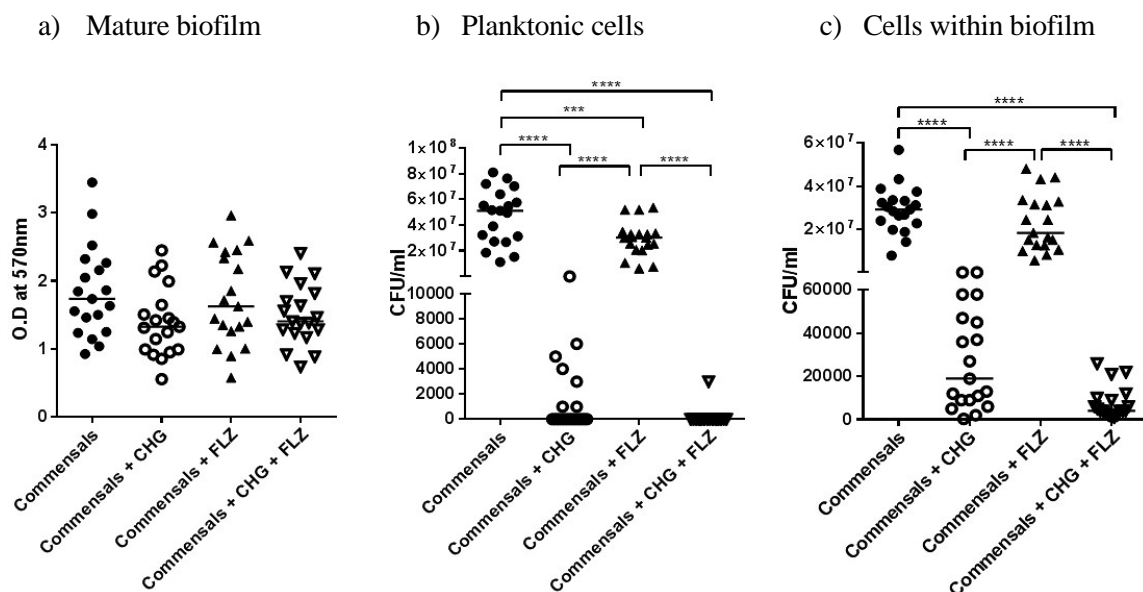


Figure 26. Thickness of mature biofilm (a) produced by colonizing vaginal *C. albicans* isolates ($n=19$) was measured after treatment of chlorhexidine digluconate (CHG) and fluconazole (FLZ) and the combination CHG + FLZ. As controls served untreated *C. albicans* from the same patients. Individual and median values are presented, depicted as optical density (OD) at 570 nm after dissolution of crystal violet. Viable count of planktonic *C. albicans* (b) and of *C. albicans* in mature biofilm (c) from asymptomatic women, commensal isolates. *** $p < 0.001$, **** $p < 0.0001$

6 DISCUSSION

6.1 DISCUSSION OF THE RESULTS

6.1.1 NO levels, symptoms and clinical findings

The pathogenesis of RVVC still remains an enigma. There has been progress in the understanding of the disease but the picture is complex with many factors to consider, such as individual differences in susceptibility to recurrent infections, diverse local immune responses and virulence factors of various candida strains. A strong inflammatory response is seen in the vaginal mucosa during relapse of RVVC. During the acute infectious episode, vaginal NO levels are significantly increased compared to after treatment as well as compared to those in healthy controls. In the current study we showed that vaginal NO concentration also correlates to clinical findings and symptoms in RVVC. It is not yet clarified if the inflammatory tissue response is the same in RVVC and sporadic VVC even though clinical findings are similar.

NO is part of the non-specific host defense and is involved in the inflammatory process. The immuno-histochemical results demonstrating iNOS expression in the mucosa indicate that the NO measured in the vagina most likely derives from epithelial iNOS. These results are supported by the inflammatory status of the vulvovaginal skin and mucosa with erythema, edema and discharge. An interesting finding was the distinct staining of iNOS in the epithelial basal membrane that has not been described before. No correlations were found between NO levels and overall iNOS staining, but the basal membrane staining may still mirror an amplified expression of iNOS in patients. Previous studies of chronic inflammatory disorders have described mucosal iNOS located in the upper part of the epithelium facing the lumen of several hollow structures [213], a finding that was replicated in our material. Increased vaginal NO concentration has been reported in a study including patients with unspecific vaginitis [207]. Therefore, to eliminate other possible influences of NO formation that could interfere with our results, we stringently excluded participants with other cervical or vaginal infections.

After antifungal therapy the vaginal NO levels in patients were decreased but still significantly higher compared to levels in healthy controls. A possible explanation of this finding might be that mucosal inflammation was still not entirely resolved.

It is still not known if formation of NO in the vagina is beneficial or harmful for the tissue. Normally, high concentrations of NO are found in the nasal airways without harming the tissue [213]. However, NO is known to have double functions and may induce damage to host cells. Destructive effects of NO likely arise from products, following the reaction of NO with oxygen radicals. In patients with active inflammatory bowel disease, the median NO concentration in rectum was ten times higher than the levels we reported in RVVC [125]. However, in unspecific vaginitis, non-infectious cystitis and asthma as well as in bacterial

infections such as cystitis and pelvic inflammatory disease, the NO levels were in accordance with our results [123, 128, 129, 207].

Women suffering from BV do not display the same inflammation in the vulvovaginal tissue and have less pain and discomfort compared to women with acute candida infection. In pregnant women with BV, nitric oxide substrates in vaginal secretions is shown to be elevated [214]. Healthy postmenopausal women with higher vaginal pH did not have higher NO concentration than healthy premenopausal women, indicating that elevated NO levels are not dependent on the pH status but rather the abnormal microbial status of the mucosa [207].

Fungal culture is the most precise method for diagnosing candida infection if wet mount is negative for hyphae. It is reported that visible yeast cells and hyphae are seen in 50% of patients with VVC [11]. Serologic tests and PCR-based diagnosis for antigen detection are currently not in clinical use, due to low reliability or too high sensitivity [12]. However, a positive culture alone does not necessarily prove that the yeast identified is responsible for the present vaginal symptoms, since 20 % of asymptomatic women are culture positive. A reliable diagnosis of vulvovaginal candidosis requires a combination of clinical findings, microscopic examination and vaginal culture that takes a few days to analyze [12].

A majority of our patients had visible hyphae and positive cultures along with typical clinical findings. A correct diagnosis of recurrent infection needing treatment is important, but is sometimes difficult to obtain. Many RVVC patients continue to be symptomatic with itch and soreness after treatment, resulting in repeated gynecological examinations and most often excessive treatment with a potential risk of generating resistant candida strains. In that aspect vaginal NO measurement could serve as a complementary and instant diagnostic tool for recurrent infections with the intention to avoid unnecessary treatment. Based on our findings, using cut-off levels of either NO 25 or 50 ppb will ensure a low risk of prescribing fluconazole unnecessarily and only a few cases that could have benefitted from treatment will be missed, *Figure 10 a*. The ROC analyses showed that a careful examination in combination with microscopy also has a high sensitivity and specificity for a correct diagnosis confirmed by positive culture. However, the testing and examinations in this study was performed by a gynecologist with special training in vulvovaginal diseases and long experience of managing RVVC. In other less specialized clinical settings, NO measurements could be of value for diagnosing relapsing infections, even more so when a microscope is not available. In asthma and interstitial cystitis, NO analyses are used for monitoring the inflammatory activity and optimizing treatment [124, 134].

6.1.2 Psoriasin in *C. albicans* infections

Several antimicrobial peptides with possible involvement in the mucosal immune response against candida were analyzed. The expression pattern was similar to a previously described expression survey of AMPs [189]. However, in that survey AMPs were analyzed in HPV positive vulvovaginal lesions inducing a different immune response in the mucosa compared

to candida infections. Psoriasin was the most up-regulated and expressed AMP during acute *C. albicans* infection. To find possible mechanistic actions of psoriasin, a pull-down assay was performed using whole *C. albicans* cells or the fungal cell wall elements, chitin, mannan and β -glucan. Psoriasin was found to bind to whole yeast cells and to β -glucan and was precipitated by *C. albicans* in the vaginal lavage samples.

The antibacterial and antifungal activity by psoriasin is still not fully identified. Our experiments, performed in a solution resembling the vaginal milieu, indicate a strong interaction between psoriasin and β -glucan. Electron microscopic investigation showed significant thinning of the *C. albicans* cell wall with reduction of β -glucan after exposure to psoriasin, demonstrating a possible interaction with the protein.

We studied two main phases in the pathogenesis of *C. albicans* infection. Adhesion and induction of cytokine response were investigated in order to examine possible consequences of psoriasin-mediated alterations of the candida cell wall. The experiment with vaginal lavage from patients with RVVC and healthy controls confirmed the presence of an anti-adhesive action. In an *in vitro* assay the anti-adhesive action was partially associated with psoriasin. However, in the lavage samples there was not a total blocking effect, indicating that additional anti-adhesins are present *in vivo*.

After adhesion of a microbe, a receptor-mediated interaction with the host cell commonly induces an immune response in the tissue. Glucans, which are important fungal PAMPs, [35] bind to psoriasin. Since β -glucan has been shown to mediate induction of pro-inflammatory cytokines [36, 37], we anticipated a reduced immune stimulation as a consequence of the β -glucan depleted cell wall of psoriasin treated candida cells. However, contrarily, in the *in vitro* experiments, the IL-8 production by vaginal epithelial cells was increased when *C. albicans* was pretreated with psoriasin. A possible explanation for this somewhat surprising result could be a reduction of the availability of soluble β -glucan in the cell wall since large β -glucan particles induce cytokine production, while soluble β -glucans act as antagonists of these processes [41]. We suggest that psoriasin might induce changes in the cell wall that amplifies exposure of large β -glucans at the cell surface. The mucosal immune protection against *C. albicans* might therefore be promoted by psoriasin mediated anti-adhesive effect and enhancement of cytokine expression.

During an acute episode of RVVC, psoriasin was explicitly upregulated, although it did still not protect the host from infection. Since we only investigated patients with RVVC we cannot rule out that patients with a single acute infection with VVC trigger a psoriasin response that is more effective compared to patients with frequent infections. It is also interesting to speculate if polymorphisms in the psoriasin gene, S100A7, might be associated with recurrent candida infections. This remains an important field to explore in the future.

6.1.3 Antimicrobial peptides in HSIL

Several mechanisms of immune evasion leading to the persistence of HPV infections have been described [10, 70, 71]. HPV do not replicate in antigen-presenting cells, do not cause lysis of keratinocytes and the infection does not cause a typical immune response with tissue damage. Thereby HPV avoid recognition by the immune cells. Despite the lack of typical immune response, HPV induce a persistent and chronic inflammation as the host can remain ignorant of the pathogen for long periods [10]. In the development of HPV associated neoplasia chronic inflammation plays an important role [180].

HIV infected women have higher incidence of HSIL and cervical cancer. Moreover, HPV infection may increase the risk to acquire HIV infection. One might speculate if HPV induce a local inflammatory response and thereby cause an impairment of the innate immunity, which could contribute to persistence of infection and thus promote HIV infection [195].

There are only a few studies addressing the role of AMPs and particular psoriasin in HPV induced precancerous and malignant lesions and the results from those studies are contradictory. Psoriasin is involved in the differentiation of keratinocytes and it promotes endothelial cell proliferation by increasing vascular endothelial growth factor (VEGF). Previous investigations on epithelial skin tumors have shown that precancerous lesions display higher expression of psoriasin compared to invasive tumors and healthy samples [191, 199]. Psoriasin expression is considered to correlate with tumor progression and is associated with poor prognosis in breast cancer and has been suggested as a candidate diagnostic marker [203, 205].

We analyzed the expression of AMPs, with special focus on psoriasin, in cervical high-grade squamous intraepithelial lesions (HSIL) before and after surgical treatment. The performed analyzes were PCR, ELISA and IHC, thereby identifying both gene and protein expression in the same patients. Other studies in this field have mainly used one method at a time. Additional strengths in our study are the clearly defined and carefully selected study groups and two independent pathologists verifying the histopathological diagnosis.

The results of our analyses in HSIL samples showed significantly lower expression of both mRNA and protein levels of psoriasin before treatment. After surgical excision the levels increased to equal levels as in healthy controls. The results diverge from results in previous investigations showing higher expression of psoriasin in precancerous lesions compared to controls [191, 199]. In benign vulvar condylomas, the expression of HBD-1-3 and psoriasin is significantly up-regulated [189]. During normal conditions in the oral mucosa, expression of psoriasin mRNA is higher than in extra-oral skin. Analyzing the protein levels showed contrasting results with a high quantity of protein retained in the keratinized epithelial layers [215]. The higher expression in the mucosa might be explained by the thinner mechanical barrier and multiple present microbes. The produced proteins may conduct their activity intracellularly or be released into the cavity. Our results indicate that psoriasin is downregulated in HPV induced HSIL which might be a part of the premalignant process, but

it can be discussed if the low levels rather are a prerequisite for persistent HPV. However, our study is small and lack samples from cervical cancer patients. There is need for larger studies to explain the role of AMPs in HPV induced pre- and malignant processes.

IL-8 was analyzed to follow the inflammatory response. The mRNA expression was significantly higher in patients with HSIL before treatment compared to after treatment. Although, on the protein level, these results could not be confirmed. Since elevated levels of cytokines are proposed to affect the transformation of persistent infection to malignant lesions these results are in line with former results of elevated IL-8 in SCC and HSIL [195, 199].

6.1.4 Biofilm and antifungal strategies

Managing the treatment of RVVC is challenging. Many patients suffer from relapses despite adequate therapy and their quality of life is severely affected. *C. albicans* is the most common pathogen in RVVC and has a high capacity to form biofilm. Therefore, one can assume that biofilm is of importance for vaginal *C. albicans* creating an environment where the fungus can hide, persist and then disperse from the biofilm and cause recurrent infections. Consequently, it is important to find alternative treatment strategies to dissolve biofilm and expose the fungus to antifungals. It is also essential to find a way to prevent formation of biofilm. Our results show that both *C. albicans* from RVVC patients and commensal strains form biofilm. The commensal strains might have a potential to become pathogenic under other circumstances.

The study analyzed the effect of fluconazole and chlorhexidine digluconate on biofilm from pathogenic and commensal strains. Since long-term administration of fluconazole is the first line treatment of RVVC [16], it is interesting to notice that our results show no effect of FLZ on preventing new biofilm formation nor any ability to dissolve already established biofilm. FLZ had the ability to reduce the planktonic candida cells but not candida growth within the biofilm, which is an important finding in the perspective of recurring infections.

Several studies have shown antibacterial and antifungal effect of CHG [216, 217]. The proposed mechanism of action is interaction with the cell wall and the effects are dose dependent with both bacteriostatic and bactericide capacity [217]. Therefore, we investigated the effect of CHG alone and in combination with FLZ in *C. albicans* growth and impact on biofilm.

CHG was able to dissolve established biofilm and treatment with CHG almost totally eradicated candida cells in the planktonic state. An essential finding was that CHG treatment also had effect on candida growth within the mature biofilm with a significant reduction of viable candida cells. We speculate that CHG might change the properties of the biofilm and thus enabling the molecules to penetrate and interact with the cell wall.

There was also a strong effect of CHG in inhibiting new biofilm formation which might be a key component of prevention and treatment of RVVC. If candida fails to produce biofilm the treatment and eradication of the pathogen will be manageable.

We speculate whether women with RVVC are colonized with candida also between symptomatic episodes. If so, these strains need to be eradicated to avoid recurrences. Based on our results, vaginal CHG might be a possible treatment option for patients with RVVC and might also be prescribed for prophylactic use to diminish recurrences. In symptomatic cases a combined treatment with both CHG and FLZ could result in improved eradication of candida. It has previously been shown that chlorhexidine is superior to fluconazole in eradicating *C. albicans* in oral candidosis [218]. However, the influence on the commensal vaginal flora and the vaginal epithelial cells still needs to be clarified.

6.2 METHODOLOGICAL CONSIDERATIONS

6.2.1 Participants

The patients were recruited from the Vulvar Clinic at Danderyd Hospital and from the national cervical screening program whereas the healthy controls were recruited through advertisements at Karolinska Institutet and Danderyd Hospital. A selection bias in Study I and II must be considered since the controls were significantly younger than the patients and the majority of the healthy controls were university students. However, a majority of the patients also had a high level of education.

Considerable effort was done to carefully exclude patients and controls with concomitant genital infections since other mucosal infections might have influenced the results in several aspects. The exclusion criteria were strictly followed. Fifty-eight women with assumptive candida infection came for a recruitment visit, but only 28 subjects fulfilled the inclusion criteria for study I, *Figure 7*. Due to methodological reasons, we could only include 16 of the patients in Study II and 18 patients in study IV. Of the 37 invited patients with cervical dysplasia, 19 patients were included, *Figure 8*. The meticulous sampling and the inclusion process are major strengths of the studies. However, one must consider the deficient selection of participants to study II and IV as weakness of the studies.

Another limitation of the studies is the shortage of menstrual data. However, it has been shown that NO levels show no significant variation during the menstrual cycle [129]. It has been described that some AMPs vary during the menstrual cycle but the cyclic regulation is abolished in oral contraceptive and levonorgestrel intrauterine device users [168]. Another weakness of Study III is the lack of samples from patients with cervical cancer which would have added valuable information in elucidating the role of AMPs in HPV induced premalignant and malignant lesions. The low number of samples is a major limitation of Study III and IV and the *in vitro* results need to be confirmed in clinical trials.

6.2.2 NO measurement

NO has previously been shown to be an indicator of inflammation in disorders of various organs. The method for NO measurement in the vagina, using the catheter balloon, was first described by Sioutas et al [207]. The method was also evaluated in the uterine cavity by the same group [208], showing that 2 min incubation of the balloon is a sufficient time to measure the NO levels. The procedure was well tolerated and had no complications.

We chose to use the chemiluminescence analysis, which is a direct method measuring the actual NO concentration. Babula et al, showed that women with RVVC, during an episode of acute culture-verified infection had lower concentrations of the NO metabolites nitrite and nitrate compared to controls, as opposed to our results [219]. Measuring NO metabolites is an indirect method and these markers in vaginal fluid are influenced by several other factors such as dietary discrepancies and the presence of commensal bacteria [220]. Since bacteria can both produce and consume nitrate and nitrite they might affect the outcome based on analyses of these substances [221].

6.2.3 Examination and sampling

Methodological aspects on the testing procedures should be considered. The methods with examination and symptom scores were designed for Study I. The scores have not previously been used and are not validated. To minimize detection bias in the examination score, the same examiner did all the scoring. Since the RVVC patients had a long history of symptoms one might consider recall bias.

Four patients had positive culture for *C. albicans* after antifungal therapy and five other patients had visible hyphae in wet mount. Culture is considered the most reliable method to identify candida infection [16] and the divergence in results of the two methods could be caused by an over diagnosis of hyphae in the microscope or a misdiagnose of fungal culture. Positive culture for *C. albicans* and symptoms of vulvovaginal candidiasis were inclusion criterion, which minimize selection bias.

The sampling of vaginal lavage for Study II was technically challenging and in some cases it was not possible to obtain any VL after the installation time of 5 minutes, because the fluid did not remain in the vagina. This was the major reason why only 16 patients were included in Study II.

The samples taken from the vaginal wall for Study I and II were on the other hand easy to obtain. The tissue was injected with local anesthetic and the sampling was well tolerated. The general mucosal inflammation seen in VVC implies that the biopsy samples are representative for inflammatory changes throughout the vagina. The representation of the samples taken from the cervix in Study III was more complicated. The samples were obtained after colposcopic identification of the suspected lesions. However, it can be technically difficult to acquire several samples from the same area of HSIL. The representation of the

samples does probably not reflect the total picture of AMPs throughout the whole cervical mucosa. The follow-up biopsies were taken from the transformation zone of the cervix.

Since the commensal strains in Study IV were randomly selected we do not have any data of the women harboring these strains and one might consider selection bias.

6.2.4 Treatment

The RVVC patients received antifungal therapy with oral fluconazole for six weeks for study purposes and in accordance with Swedish guidelines at the time of the study. After treatment, four patients had positive cultures which is in line with the pathogenesis of RVVC with high recurrence rate of the infections. The length of the treatment period might have been too short to eliminate growth of *C. albicans* in all patients, alternatively the fluconazole dose was too low. These results demonstrate that long-term treatment is needed as previously presented and that the dose needs to be individualized. The recommended long-term treatment is fluconazole, with induction of 150 mg every 72 h for 3 doses and thereafter maintenance regimen with 150 mg weekly for 6 months [16].

If the candida species are resistant to fluconazole the treatment has to be adapted to the susceptibility test results. Oral treatment with itraconazole or local treatment with clotrimazol, econazole or boric acid can be used. Local steroids in combination with antifungal treatment is sometimes used during acute infection to relieve symptoms of the vulva.

6.2.5 Chemicals and reagents

Vaginal fluid simulant was used as a medium to mimic the vaginal milieu. Original vaginal secretion includes fluid from the upper reproductive tract such as endometrial and tubal fluids, cervical mucus, vaginal transudate and exfoliated epithelial cells. The VFS used in our study is intended to have the same properties as the secretion originating in the vagina of non-pregnant, premenopausal and healthy women. The formulation of VFS has been proved useful in other research of contraceptive and drug administration [212].

Chlorhexidine digluconate as an antiseptic for vaginal use has only been limitedly explored [222]. Since CHG has a broad effect on microbes one must consider the influence on the commensal bacterial flora. Al-Niaimi et al showed that use of 2% chlorhexidine gluconate as vaginal preoperative preparation was not associated with increased vaginal irritation or allergic reactions [223]. When treating women with intravaginal 0.5% chlorhexidine gluconate gel for 7 days the tolerability was good [222].

6.3 FUTURE PERSPECTIVES AND CLINICAL IMPLICATIONS

Vaginitis is amongst the most common clinical problems in women of childbearing age, annually accounting for millions of gynecological office visits worldwide. RVVC causes excessive suffering and affect the patients' sexual health and quality of life. Moreover, RVVC is a known risk factor for development of vulvar pain and dyspareunia and it is suggested that the repeated inflammation induces hypersensitivity and hyperinnervation of the vulvovaginal mucosa. A possible reduction of the inflammatory response during RVVC might be of great importance to decrease that risk. In treatment of other chronic inflammatory conditions such as asthma, reduction of inflammation has priority over cure. Accordingly, complementary treatment to reduce the inflammatory tissue response in patients with RVVC might be of value. Since a correct diagnosis of RVVC can be difficult to obtain, NO measurement might be a useful complementary diagnostic tool, in some settings. Over consumption of fluconazole, which might lead to antifungal resistance in the long term could thus be avoided. It would be of interest to study NO levels in other gynecological conditions with affected immune response, such as BV. BV often coexist with candida and although it does not cause visible inflammation in the vaginal mucosa some symptoms overlap with candida vulvovaginitis.

There is great need for new treatment options and prevention strategies for vulvovaginal candida infections. Recurrences are a major clinical difficulty and there is a lack of useful prophylactic approaches. Since biofilm is a major part of candida virulence, new approaches in antifungal therapies focusing on biofilm eradication would be valuable. As long as the antifungal treatment does not affect the candida cells in the biofilm, possibility of resolving the infection is low. Treatment aiming to dissolve biofilm and kill the candida cells within the biofilm would be an ideal approach. Our results indicate such an action by chlorhexidine digluconate. CHG is easy to obtain, inexpensive and formulations for vaginal use have previously been described. However, to evaluate and compare the effect with other antifungal preparations, randomized, blinded treatment studies have to be conducted. It would also be of great interest to analyze the effect of CHG-fluconazole combination treatment and to evaluate possible prophylactic effects. Also, it is essential to ensure there are no harmful effects on the normal vaginal flora which has a key function for vaginal health. The effect on vaginal epithelial cells needs to be investigated to identify potential unwanted side effects.

Furthermore it still remains for future research to further reveal the pathogenesis of RVVC and identify additional mechanisms leading to candida persistence, which is a mechanism for cells to hide from immune cells and antifungal treatment.

Moreover the mechanisms of AMPs in inflammatory response and carcinogenesis need to be further explored both in animal models and humans before the results can be applied clinically. Psoriasin has been shown to take part in the immune response and have multiple functions. It may be beneficial for the host but contradictory it might also be involved in tumor progression. Psoriasin has been suggested as part of a panel of clinical biomarkers,

aimed at diagnostics and mapping of epithelial malignant conditions such as breast and bladder cancer.

Finding a possible clinical use for antimicrobial peptides against skin and mucosal infections is a future possibility. Synthetic and non-toxic topical formulas of AMPs as treatment or prevention of infections could be an interesting prospect. However, more knowledge is needed in this field since synthetic AMP derivatives previously designed have shown cytotoxicity such as hemolysis. The probability of unintended leakage into systemic circulation must be considered as well as the risk of potentiating the neoplastic development, since continuous presence of AMPs might trigger the inflammatory process.

The results of this thesis emphasize that the innate immune system plays as an important role in recurrent gynecological infections. If targets of innate immunity are found, they might be possible objectives for future treatment, as an alternative to current pharmacological antimicrobial treatment. Future discoveries of possibilities to strengthen the local immune defense to prevent microbes from invading tissues would also be of great importance.

7 CONCLUSIONS

Innate immune response is induced by RVVC and HPV infections of the lower female genital tract. A better understanding of local innate immunity is important to enable development of future preventive and therapeutic strategies for these infections.

- NO is significantly elevated in patients with acute episodes of RVVC and decreases after antifungal treatment. Results illustrate that the pronounced inflammatory response in the tissue during infection correlates to the patients' symptoms. iNOS expression in the mucosa suggest that NO is produced by epithelial cells.
- A novel mechanistic action of psoriasin was identified, demonstrating that psoriasin has an anti-adhesive effect on *C. albicans*. Further, psoriasin affects the proinflammatory response in the epithelial cells.
- In HPV induced cervical highgrade squamous intraepithelial lesions, the gene expression and protein levels of the antimicrobial peptide psoriasin were lower before treatment. Six months post conisation, levels were elevated and comparable to those in healthy controls with a corresponding reduction of IL-8 mRNA expression.
- Chlorhexidine digluconate inhibits biofilm formation and dissolves already established, mature, biofilm. In addition, chlorhexidine digluconate eradicates *C. albicans* both in planktonic phase and within biofilm. Although fluconazole is effective against planktonic *C. albicans*, it has no effect on biofilm or candida cells within the biofilm.

8 POPULÄRVETENSKAPLIG SAMMANFATTNING

Faktorer i det lokala immunförsvaret som påverkas vid upprepade och kvarstående gynekologiska infektioner

Bakgrund

Gynekologiska infektioner är mycket vanliga och orsakas av olika bakterier, virus, svampar och parasiter. Vissa kan ge långdragna och återkommande besvär som i många fall leder till stort lidande, försämrad sexualitet och sänkt livskvalitet. De vanligaste symtomen är flytningar, rodnad, klåda, smärta, blödning och samlagssmärta. Merparten av infektionerna, men inte alla, är sexuellt överförbara. Sjuttiofem procent av alla kvinnor får svampinfektion någon gång i livet och 6-9% drabbas av återkommande svampinfektioner (RVVC), definierat som 3-4 infektioner/år. Dessa kvinnor är oftast helt friska i övrigt och orsakerna till de återkommande infektionerna är oklara och anses multifaktoriella. RVVC är i regel svårbehandlat och medför ofta konstanta lokala besvär hos kvinnan. Vid upprepade infektioner finns det en ökad risk för utveckling av vestibulodyn, ett smärttillstånd runt slidöppningen som i många fall omöjliggör samlag.

Humant papillomvirus (HPV) är den vanligaste sexuellt överförda infektionen i världen och majoriteten av dessa infektioner läker ut spontant. Det finns över 200 varianter av HPV som delas in i låg- och högrisk typ med avseende på deras förmåga att inducera cellförändringar och cancerutveckling. Kvarstående HPV infektion kan orsaka cancer i livmoderhalsen, vagina, vulva, analkanalen, penis, mun och svalg. Livmodertappens yta är särskilt känslig för infektionen. I transformationszonen där två olika typer av slemhinnor möts, finns det stor risk att virusgenomet inkorporeras i värdcellens DNA. Cellförändringarna delas in i olika grader och förändringarna kan upptäckas i tidigt skede med den screeningverksamhet med cellprovtagning som finns i många länder. I och med genomförandet av screening i Sverige, har antalet fall av livmoderhalscancer minskat betydligt.

Orsakerna till varför en del infektioner inte läker ut eller återkommer gång på gång hos vissa individer är oklara. Man har hittills inte kunnat hitta någon säker förklaring varken i det medfödda eller i det specifika immunförsvaret. Det medfödda lokala immunförsvaret är komplext och består av många olika komponenter som har stor betydelse för infektionernas läkningsprocess. Kvävemonoxid (nitric oxide (NO)), antimikrobiella peptider (AMP) och cytokiner är alla viktiga faktorer i det medfödda immunförsvaret som riktas mot invaderande mikroorganismer. Det saknas idag fortfarande mycket kunskap om hur dessa komponenter påverkar den inflammatoriska processen vid infektioner och hur kopplingen till cancerutveckling ser ut.

Målsättningar

Våra huvudmålsättningar var att undersöka NO, AMP och cytokiner hos kvinnor med upprepade vulvovaginala svampinfektioner och i HPV inducerade precancerösa förändringar på livmodertappen med syfte att öka förståelsen av det medfödda immunförsvarets roll vid dessa infektioner.

Specifika målsättningar

- Att mäta NO koncentrationen i vagina samt att studera uttrycket av enzymet iNOS i vaginala biopsier före och efter behandling av en akut episod av återkommande vulvovaginal candidainfektion och att jämföra med friska kontroller. Att korrelera NO koncentrationen till symtom och till den kliniska bilden.
- Att undersöka interaktionen mellan svampen *C. albicans* och det lokala försvaret i vaginas slemhinna, med avseende på antimikrobiella peptider vid RVVC och att förstå den antimikrobiella peptiden psoriasins roll i slemhinnans försvar mot *C. albicans*.
- Att studera uttrycket av antimikrobiella peptider och cytokinet IL-8 i vävnad hos patienter med precancerösa förändringar på livmodertappen före och efter behandling och jämföra med friska kontroller.
- Att undersöka effekten av klorhexidindiglukonat på *C. albicans* och dess biofilm från patienter med RVVC och att jämföra effekten med flukonazol, den vanligaste behandlingen vid RVVC.

Material och Metoder

I studierna ingick 28 patienter med RVVC, 19 patienter med höggradiga cellförändringar på livmodertappen och 45 friska kontroller. Alla deltagare genomgick en noggrann undersökning för att utesluta andra infektioner som skulle kunna påverka resultaten. Hos patienterna med svampinfektion mättes NO koncentrationen i vagina före och efter behandling och vävnadsprover togs från slemhinnan i vagina. Dessa jämfördes med prover från friska kontroller. I vävnadsproverna analyserades förekomst och mängd av antimikrobiella peptider samt förekomsten av enzymet iNOS som styr bildandet och frisättningen av NO. Peptiden psoriasin visade sig intressant och vi studerade dess funktion i det lokala försvaret mot *C. albicans* med ett flertal olika analysmetoder.

Patienterna med cellförändringar genomgick provtagning av livmodertappen och kirurgisk behandling. I vävnadsprover analyserades förekomst och mängd av antimikrobiella peptider på samma sätt som hos patienterna med svamp.

Behandlingseffekten av klorhexidindiglukonat och flukonazol studerades hos *C. albicans* stammar från patienter med upprepade infektioner samt stammar från symtomfria kvinnor. Effekten avseende nybildning av biofilm, förmåga att bryta ner etablerad biofilm och avdödande av candida celler studerades.

Resultat

NO i vagina har en kraftigt förhöjd koncentration hos patienter med en akut episod av RVVC. Efter behandling sjunker koncentrationen betydligt men kommer fortfarande inte ner i samma låga nivå som hos friska kontroller. NO koncentrationen korrelerar väl till graden av symptom och kliniska fynd. iNOS kunde identifieras i epitelcellslagret i högre andel av patienterna jämfört med de friska kontrollerna.

Den antimikrobiella peptiden psoriasin ökar signifikant vid infektion med *C. albicans*. Psoriasin binder till β -glucan som är en beståndsdel i cellväggen hos *C. albicans*. Där påverkar den cellväggens tjocklek och minskar svampens vidhäftningsförmåga till vävnaden. Psoriasin tillsammans med *C. albicans* påverkar det proinflammatoriska svaret i epitelet.

Proteinkoncentrationen och gen uttrycket av den antimikrobiella peptiden psoriasin hos patienter med HPV inducerade precancerösa cellförändringar var låga före behandling och ökade signifikant sex månader efter kirurgisk behandling. Samtidigt sågs en minskning av genuttrycket av den proinflammatoriska cytokinen IL-8 efter behandling. Analys av proteinnivåerna kunde dock inte verifiera denna skillnad relaterad till behandling.

I våra cellstudier har klorhexidindiglukonat god effekt mot *C. albicans* och dess biofilm. Vi visar att substansen påverkar både nedbrytning och nybildande av biofilm. Klorhexidindiglukonat är dessutom mer effektiv än flukonazol, den rekommenderade behandlingen vid RVVC, på att avdöda candida celler som finns inne i biofilmen.

Slutsats

Resultaten visar att det medfödda immunförsvaret spelar en viktig roll vid RVVC och vid HPV inducerade cellförändringar på livmodertappen. NO nivåer speglar det inflammatoriska svaret i vävnaden vid akuta episoder av RVVC och korrelerar väl till den kliniska bilden och patientens symptom. Våra resultat visar en ny funktion hos den antimikrobiella peptiden psoriasin som motverkar att candida fäster till vävnaden och på så sätt försvårar utvecklingen av infektionen.

Det finns ett stort behov av nya strategier för preventiva åtgärder och behandlingsmetoder vid RVVC. De behandlingar som finns idag är inte alltid effektiva och många patienter får täta återfall när behandlingen avslutas. I våra analyser visar vi att klorhexidindiglukonat kan vara

ett alternativ för att förbygga och behandla infektioner med *C. albicans*, men resultaten behöver bekräftas i kliniska behandlingsstudier.

Psoriasin koncentrationerna och det proinflammatoriska svaret i vävnaden påverkas även vid HPV inducerade precancerösa förändringar på livmodertappen. Betydelsen av detta fynd är fortfarande oklar, men tidigare studier av andra cancerformer har visat att det finns samband mellan psoriasin och utveckling av viss cancer. Framtida forskning behöver fortsätta undersöka antimikrobiella peptiders roll vid den maligna processen där psoriasin eventuellt kan fungera som markör och prognostisk faktor.

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